ISSN: 2667-4203

ESKİŞEHİR TECHNICAL UNIVERSITY JOURNAL OF SCIENCE AND TECHNOLOGY
C– Life Sciences and Biotechnology

ESKİŞEHİR TEKNİK ÜNİVERSİTESİ BİLİM VE TEKNOLOJİ DERGİSİ
C – Yaşam Bilimleri ve Biyoteknoloji

Volume / Cilt 14 Number / Sayı 2 July / Temmuz - 2025

ESKİŞEHİR TEKNİK ÜNİVERSİTESİ BİLİM VE TEKNOLOJİ DERGİSİ
C- YAŞAM BİLİMLERİ VE BİYOTEKNOLOJİ

Eskişehir Technical University Journal of Science and Technology
C -Life Sciences and Biotechnology

Estuscience – Life

Volume: 14 / Number: 2 / July - 2025

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ISSN: 2667-4203

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Volume: 14 / Number: 2 / July – 2025

Owner / Publisher: Prof. Dr. Adnan ÖZCAN (Rector) for Eskişehir Technical University

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ABOUT

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Books

Haupt RL, Haupt SE. Practical Genetic Algorithms. 2nd ed. New York, NY, USA: Wiley, 2004.
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Eskişehir Technical University Journal of Science and Technology
C -Life Sciences and Biotechnology

Estuscience – Life, 2025, 14 (2) pp. 44-58, DOI: 10.18036/estubtdc.1536495

RESEARCH ARTICLE

IN-VITRO ANTIBIOFILM ACTIVITY AND GROWTH INHIBITORY EFFECTS OF *Origanum onites* ESSENTIAL OIL AND CARVACROL AGAINST *Escherichia coli* ATCC 25922 AND METHICILLIN-RESISTANT *Staphylococcus aureus* (MRSA) ATCC 43300

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Abstract

The treatment of infections caused by *Escherichia coli* and Methicillin-resistant *Staphylococcus aureus* (MRSA) has become difficult due to the increasing development of resistance to the antibiotics used. Nowadays, in the fight against these infections, the importance of natural and inexpensive plant essential oils and their bioactive molecules with known antibacterial activity has been increasing. This study aimed to investigate the antibacterial and antibiofilm effects of *Origanum onites* essential oil (OEO) and carvacrol, to which bacteria cannot develop resistance, as an alternative to antibiotics. The chemical content of OEO was analyzed by GC-MS system. Antibacterial activity was analyzed by disk diffusion, macro broth dilution, and antibacterial curve assays, and also antibiofilm activity was analyzed by the quantitative crystal violet method. Carvacrol was defined as the major component in the OEO composition. The results showed that OEO and carvacrol exhibited antibacterial activity against *E. coli* and MRSA with the minimum inhibition concentration (MIC) of 100 µg/mL and 50 µg/mL, respectively. The antibacterial curve assay results showed that OEO and carvacrol exhibited bactericidal activity against *E. coli* and MRSA. OEO and carvacrol inhibited the biofilm formation of *E. coli* and MRSA in the range of 15.5%-80.7% at MIC, 1/2MIC, and 1/4MIC concentrations.

Keywords

Escherichia coli,
Origanum onites,
Antibiofilm activity,
Carvacrol,
Methicillin-resistant
Staphylococcus aureus

Time Scale of Article

Received : 21 August 2024
Accepted : 26 February 2025
Online date : 25 July 2025

1. INTRODUCTION

Escherichia coli (*E. coli*) is largely found in the flora of the digestive tract and constitutes the largest proportion of Gram-negative facultative anaerobic bacteria in the intestines. As an opportunistic pathogen, it is the most frequent causative agent in urinary tract infections and community-acquired bacteremia [1]. It causes bloody diarrhea in the intestine. Apart from the intestine; it also causes urinary tract infections, neonatal meningitis, pneumonia, septic arthritis, skin and soft tissue infections [2]. Methicillin-resistant *Staphylococcus aureus* (MRSA) is a Gram-positive and facultative anaerobic bacterium that causes serious hospital acquired infections and threatens public health worldwide. MRSA infections are generally resistant to many antibiotics [3]. Diseases caused by pathogenic *E. coli* and MRSA strains in humans are generally treated with antibiotics, but the resistance developed by the strains against antibiotics increases the duration and cost of treatment, and sometimes the treatment fails. Unconscious approaches such as using the wrong dose of antibiotics, using antibiotics out of necessity, and not using the right antibiotics for treatment cause pathogenic bacteria to acquire multiple antimicrobial resistance instead of killing them. Although most of the resistance mechanisms developed by the *E. coli* and MRSA species against antimicrobials are known, an antimicrobial that can overcome

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all these mechanisms has not yet been developed [4]. Due to increasing resistance, it is now understood that the fight against pathogenic bacteria cannot continue with classical antibiotics. Therefore, it is necessary to develop alternatives to antimicrobials to which bacteria cannot rapidly develop resistance and to make them available for the treatment of bacterial infections as soon as possible. The fact that bioactive compounds in the composition of essential oils obtained from plants show antimicrobial and antibiofilm effects has increased the interest in essential oils in recent years [5, 6]. In addition, it is thought that they do not have toxic effects due to their natural structure and the development of bacterial resistance to chemicals in essential oils becomes difficult [7]. Essential oils can be an adjuvant and/or alternative to antibiotics because they do not harm human health and are both cheap and easily accessible.

There are 28 *Origanum* species in Turkey. *Origanum onites* are among the important export products of Turkey. *Origanum* species growing in Turkey are used as spices, condiments, and folk remedies. It has many uses due to its stomachic, sedative, antimicrobial, antitumor, antioxidant, antiseptic, antihelminthic, cardiovascular, and stimulant properties [8].

This study aimed to investigate the antibacterial and antibiofilm activity of the *O. onites* essential oil (OEO) and its bioactive compound (carvacrol) against *E. coli* and MRSA.

2. MATERIALS AND METHODS

2.1. Essential Oil

The OEO used in this study was obtained commercially which was obtained from *O. onites* leaves (country of origin; Turkey) by water vapor distillation. The OEO was dissolved in 10% dimethyl sulfoxide (DMSO) to prepare a 10 mg/mL stock solution. It was sterilized by filtration with a 0.22 µm Millipore filter.

2.2. Test Microorganism

Escherichia coli (ATCC 25922) reference bacterial strain and Methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC 43300) reference strain were used in this research.

2.3. Antimicrobial Agent

Carvacrol (Sigma-Aldrich, W224511, ≥ 98.5% purity) was dissolved in 10% DMSO to prepare 10 mg/mL stock solution and then was sterilized by filtration by 0.22 µm Millipore filter.

2.4. Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

The chemical content of the OEO was analyzed by Shimadzu QP 5050 (Kyoto, Japan) GC-MS system. Varian CP WAX 52 CB capillary column (50 m x 0.32 mm ID, df: 1.2 µm) was used as the separation column. Helium (99.999%) was used as carrier gas with a constant column inlet pressure of 10 psi (1 psi = 6894.76 Pa). A volume of 0.01 mL was injected into the device. The GC oven was programmed as follows: The initial column temperature was set at 60^0C, maintained constant for 1 min, then increased to 220^0C with a temperature increase of 2^0C/min, and maintained constant at the final temperature of 220^0C for 20 min. Library searches were performed using Nist, Wiley, and Tutor libraries. The ion source temperature and injection block temperatures were set to 250 and 280^0C, respectively. The emission current of the ionization filament was set to an energy of 70 eV.

2.5. Disk Diffusion Method

The antibacterial activity of the OEO and carvacrol was determined *in vitro* using the disk diffusion method according to Clinical Laboratory Standards Institute Standards (CLSI) [9]. The OEO and carvacrol were prepared at 10, 2, 1, 0.5, and 0.25 mg/mL concentrations. *E. coli* and MRSA were incubated in Mueller Hinton Broth (MHB) medium at 37°C for 18-24 h and the turbidity of the prepared suspensions was adjusted to 0.5 McFarland ($1x10^8$ CFU/mL). *E.coli* and MRSA suspension (0.1 mL) were taken and sown on Mueller Hinton Agar (MHA) medium according to the spreading plate method and allowed to dry for 5-10 min. Sterile blank disks (6 mm diameter) were impregnated with 20 µL of the OEO and carvacrol prepared at five different concentrations. The 10% DMSO-impregnated disk was used to see the solvent effect (also as the negative control) and Gentamicin (10 µg, Oxoid) antibiotic disk was used as the positive control. The disks were placed on the surface of the agar medium using sterile forceps. The media were incubated in an incubator at 35°C for 24 h. After incubation, the zone diameter (mm), which indicates the zone of inhibition, was measured.

2.6. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The OEO and carvacrol showing an inhibition zone in the disk diffusion test were further tested to determine the MIC values by broth macro-dilution dilution method according to the recommendations of CLSI [10]. The turbidity of the bacterial suspensions, which were incubated in MHB medium at 37°C for 18-24 h, was adjusted to 0.5 McFarland ($1x10^8$ CFU/mL). 2 mL of stock OEO and carvacrol were added to the first test tubes containing 10% DMSO, and two-fold serial dilutions were prepared at a concentration range of 800-6.25 µg/mL. An equal volume of bacteria was added to the test mediums to make the final density of the bacteria per tube $\sim 5x10^5$ CFU/mL. Thus, the final concentrations of the OEO and carvacrol in the test tubes, diluted at a ratio of 1:2, were 400-3.125 µg/mL. Positive controls (bacteria + medium) and negative controls (medium alone and the essential oil alone) were also established. The lowest concentrations of the OEO and carvacrol that inhibited the visible growth of bacteria after overnight incubation at 37°C were recorded as the MIC values. MBC is the lowest concentration of antimicrobial required to kill a particular bacterium. To determine the MBC values, 0.1 mL of the dilutions without bacterial growth were spread on MHA media and incubated at 37°C for 24 h. At the end of the incubation, the OEO and carvacrol concentrations in the Petri dishes, where there was no bacterial growth, were recorded as the MBC values [11].

2.7. Antibacterial Curve Assay

The MIC and 1/2MIC values of the OEO and carvacrol were selected as the test concentrations. The OEO and carvacrol were added to the MHB mediums at the determined concentrations. Bacterial inoculums, adjusted to 0.5 McFarland, were added to the tubes to achieve a suspension containing $\sim 5x10^5$ CFU/mL. A tube containing medium and bacteria ($\sim 5x10^5$ CFU/mL) was also prepared as a growth control. The tubes were incubated at 35ºC at 120 rpm in a rotary incubator shaker. At certain interaction times (0, 4, 8, 12, 16, 20, and 24 h), 0.1 mL was taken from each tube and diluted in physiological saline at 1/10, 1/100, and 1/1000 rates. From each dilution, 0.1 mL was taken and spread on blood agar. The media was incubated at 35ºC for 18-24 h. Then, the colonies were counted and the number of viable bacteria (CFU/mL) was determined for each incubation period. The arithmetic means and the logarithms of the viable bacterial counts in the experimental and control series were calculated. The interaction times and the $\log_{10}$ values of the corresponding viable bacterial counts are shown in the graph.

2.8. Biofilm Assay

The effect of the OEO and carvacrol on biofilm formation was analyzed by using polypropylene plastic tubes and modifying the method reported by Gómez-Sequeda et al. [12]. *E. coli* and MRSA were grown

in Luria Bertani Broth medium at 37ºC for 16-18 h. The effect of the OEO and carvacrol on biofilm formation was tested at subMIC (1/2MIC, 1/4MIC, and 1/8MIC) concentrations. Positive control (bacteria + medium) and negative controls (medium alone and essential oil alone) were also established. Approximately 5×10^5 CFU/mL *E. coli* and MRSA suspensions interacted with the OEO and carvacrol (1/2MIC, 1/4MIC, and 1/8MIC final concentrations) and incubated at 37°C for 48 h. At the end of 24 h, the contents of the plastic tubes were poured out and washed 3 times with sterile saline (0.9%) and after the washing process was completed, the tubes were dried in an oven at 60°C for 45 min. Each tube was stained with 0.2 mL of 0.4% crystal violet and incubated for 15 min at room temperature. The tubes were then washed 3 times with 0.9% sterile saline solution to remove excess stain. After washing, 0.2 mL of 95% ethanol was added to the tubes. After 15 min, the absorbance values of the samples were read at 595 nm. Finally, the inhibition percentages of each of the OEO and carvacrol concentrations were calculated using the formula below.

Inhibition percentage= 100 x ($OD_{negative\ control} - OD_{experimental}$) / $OD_{negative\ control}$)

Statistics

All tests were performed in triplicate. All data obtained in the study were analyzed using the "SPSS (Statistical Package for the Social Sciences Inc., Chicago, IL, USA) 16.0" statistical program. The data were presented as arithmetic mean ± standard deviation. The conformity of the data to normal distribution was determined by the Shapiro-Wilk test. "One Way ANOVA" was applied to test the differences and $p<0.05$ was accepted as a statistical significance level.

3. RESULTS

3.1. GC-MS Analysis

The mass percentages and retention times of the components obtained as a result of GC-MS analysis of the OEO are given in Table 1. A total of 44 compounds were identified in different percentages. It was seen that the highest value belongs to carvacrol with 53%; followed by linalool with 13.05%, and p-cymene with 12.64% rate. The peak numbers in this table are also shown in the chromatogram in Figure 1.

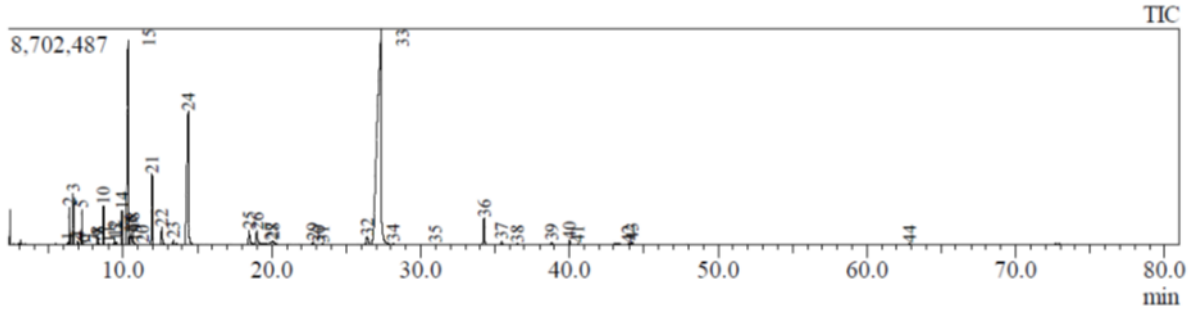


Figure 1. Chromatogram of the *O. onites* essential oil by GC-MS analysis. The x-axis represents the retention time and the y-axis represents the abundance.

Table 1. GC-MS analysis of *O. onites* essential oil

Peak	Retention time	Component	% Ratio
1	6.296	Tricyclene	0.07
2	6.381	α-Thujene	1.30
3	6.644	α-Pinene	1.82
4	6.968	Bicyclo[3.1.0]hex-2-ene, 4-methylene-1-(1-methylethyl)-	0.12
5	7.212	Camphene	1.28
6	7.339	Verbenene	0.03
7	8.044	Sabinene	0.01
8	8.248	2-β-Pinene	0.34
9	8.309	1-Octen-3-ol (CAS) Oct-1-en-3-ol	0.25
10	8.693	β-Myrcene	1.52
11	9.046	3-Octanol (CAS) n-Octan-3-ol	0.03
12	9.430	l-Phellandrene	0.23
13	9.552	Delta-3-Carene	0.12
14	9.922	α-Terpinene	1.54
15	10.335	p-Cymene	12.64
16	10.508	Bornylene	0.49
17	10.569	β-Phellandrene	0.30
18	10.656	Eucalyptol (1,8-Cineole)	0.57
19	10.820	cis-Ocimene	0.06
20	11.338	B-Ocimene Y	0.02
21	11.957	γ-Terpinene	3.37
22	12.595	trans-Sabinene hydrate	1.10
23	13.381	α-Terpinolene	0.24
24	14.379	Linalool	13.05
25	18.464	Borneol L	0.97
26	19.002	4-Terpineol	0.92
27	19.945	B-Fenchyl Alcohol	0.17
28	20.085	B- Fenchyl Alcohol	0.33
29	22.755	Benzene, 1-methoxy-4-methyl-2-(1-methylethyl)-	0.16
30	23.146	Pulegone	0.02
31	23.434	2-Cyclohexen-1-one, 2-methyl-5-(1-methylethenyl)-, (R)-	0.05
32	26.409	Thymol	0.96
33	27.363	Carvacrol	53.00
34	28.179	2-Ethyl-5-N-propylphenol	0.05
35	30.994	Phenol, 5-methyl-2-(1-methylethyl)-, acetate	0.02
36	34.274	Caryophyllene	1.82
37	35.449	Aromadendrene	0.23
38	36.492	α-Humulene	0.07
39	38.808	Viridiflorene	0.14
40	40.014	Bisabolene <beta->	0.23
41	40.561	Cadinene <delta->	0.04
42	43.981	Spathulenol	0.05
43	44.205	(-)-Caryophyllene oxide	0.19
44	62.895	4a-methyl-1,2,3,4,4a,5,6,7-octahydronaphthalene	0.08
Total			100.00

The highest peak value seen in the chromatogram belongs to carvacrol and is peak number 33. The molecular structure of carvacrol is shown in Figure 2.

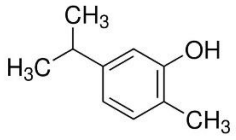


Figure 2. Molecular structure of carvacrol

3.2. Antibacterial Activity Test

In vitro antibacterial activities of the OEO and carvacrol were tested by the disk diffusion method. The zone diameter of the gentamicin antibiotic disk was measured as 23.00±1.00 mm and 24.00±1.00 against *E. coli* and MRSA, respectively. The 10% DMSO showed no inhibitory effect on *E. coli* and MRSA. For better determination of the antibacterial effect, stock OEO and carvacrol were diluted with 10% DMSO. The OEO and carvacrol were formed 48.23±0.50 mm, 45.80±1.77 mm, 38.73±1.38 mm, 30.06±0.40 mm, 22.06±1.44 mm and 49.96±2.45, 45.73±2.70, 39.26±2.80, 33.30±0.62, 24.00±1.58 inhibition zones against *E. coli* at 10, 2, 1, 0.5 and 0.25 mg/mL concentrations, respectively. The OEO and carvacrol were formed 52.70±0.26 mm, 47.03±1.68 mm, 41.00±1.22 mm, 34.23±0.66 mm, 22.00±1.60 mm and 54.36±0.77, 48.23±1.16, 42.33±0.97, 35.56±1.15, 23.33±1.13 inhibition zones against MRSA at 10, 2, 1, 0.5 and 0.25 mg/mL concentrations, respectively (Table 2).

3.3. Determination of MIC and MBC

The MIC values of the OEO and carvacrol, which were designated to show antibacterial activity against *E. coli* and MRSA. The lowest concentrations of the OEO that visually inhibited the growth of *E. coli* and MRSA were determined as 100 µg/mL and 50 µg/mL, respectively. The lowest concentrations of the carvacrol that visually inhibited the growth of *E. coli* and MRSA were determined as 100 µg/mL and 50 µg/mL, respectively (Table 3).

A 0.1 mL aliquot was taken from the tubes in which no visual growth was observed in the MIC test and spread inoculated on MHA medium. After incubation at 37°C for 24 h, OEO and carvacrol concentration in the Petri dishes without bacterial growth was determined as MBC. The MBC values of OEO against *E. coli* and MRSA were determined as 200 and 100 µg/mL, respectively. The MBC values of carvacrol against *E. coli* and MRSA were determined as 200 and 100 µg/mL, respectively (Table 4).

Table 2. Antimicrobial activity of *O. onites* essential oil and carvacrol.

	Dilution rates (w/v)	***E. coli***	**MRSA**
***O. onites* essential oil**	10 mg/mL	48.23±0.50**	52.70±0.26**
	2 mg/mL	45.80±1.77**	47.03±1.68**
	1 mg/mL	38.73±1.38**	41.00±1.22**
	0.5 mg/mL	30.06±0.40**	34.23±0.66**
	0.25 mg/mL	22.06±1.44ns	22.00±1.60ns
Carvacrol	10 mg/mL	49.96±2.45**	54.36±0.77**
	2 mg/mL	45.73±2.70**	48.23±1.16**
	1 mg/mL	39.26±2.80**	42.33±0.97**
	0.5 mg/mL	33.30±0.62*	35.56±1.15**
	0.25 mg/mL	24.00±1.58ns	23.33±1.13ns
Gentamicin		23.00±1.00	24.00±1.00
DMSO		-	-

The diameter of the zone of inhibition is expressed in millimeters. Values are presented as means±SD. Gentamicin (10 µg) antibiotic disk was used as the positive control. DMSO, Dimethyl sulfoxide was used as the negative control. Inhibition zones include the disk diameter (6 mm). *p≤0.01 compared with the positive control, **p≤0.0001 compared with the positive control, ns: non-significant (p≥0.05).

Table 3. MIC values of *O. onites* essential oil and carvacrol

	Dilution rates of *O. onites* essential oil (µg/mL)										
	400	200	100	50	25	12.5	6.25	3.125	PC	NC1	NC2
E. coli	-	-	-	+	+	+	+	+	+	-	-
MRSA	-	-	-	-	+	+	+	+	+	-	-
	Dilution rates of carvacrol (µg/mL)										
	400	200	100	50	25	12.5	6.25	3.125	PC	NC1	NC2
E. coli	-	-	-	+	+	+	+	+	+	-	-
MRSA	-	-	-	-	+	+	+	+	+	-	-

+, growth present; -, no growth (bactericidal); PC, positive control (bacteria + medium); NC1, negative control1 (medium alone); NC2, negative control2 (essential oil alone).

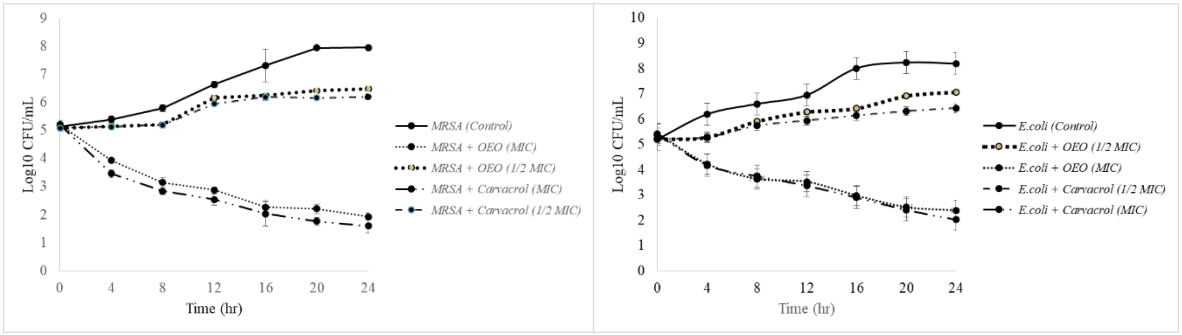
Table 4. MBC values of *O. onites* essential oil and carvacrol

	Dilution rates of *O. onites* essential oil (µg/mL)						
	50	100	200	400	PC	NC1	NC2
E. coli	nd	++	-	-	+++	-	-
MRSA	+	-	-	-	+++	-	-
	Dilution rates of carvacrol (µg/mL)						
	50	100	200	400	PC	NC1	NC2
E. coli	nd	+	-	-	+++	-	-
MRSA	+	-	-	-	+++	-	-

-, no growth (bactericidal); +, low growth; ++, moderate growth (bacteriostatic); +++, high growth (no antibacterial potential); PC, positive control (bacteria + medium); NC1, negative control1 (medium alone), NC2: negative control2 (essential oil alone); nd: not determined.

3.4. Antibacterial Curve Assay

The OEO and carvacrol prepared at MIC and 1/2MIC concentrations interacted with bacteria and their growth inhibitory effects were investigated. The arithmetic averages of the values of the number of viable bacteria at each interaction time were calculated. These values were accepted as the number of viable bacteria changing against time and $\log_{10}$ values were found. The same procedures were applied to the results in the control series (Figure 3A, B). The initial inoculums for *E. coli* and MRSA were determined as $1.6x10^5$ and $1.4x10^5$ CFU/ml. As a result of this assay, it was observed that the bacteria remained viable from 0 to 24 h with gradually decreasing amounts. When OEO interacted with *E. coli* at MIC and 1/2MIC concentrations, the number of viable bacteria at the end of the 24-hour was calculated as $2.9x10^2$ and $1.1x10^7$ CFU/mL, respectively. When carvacrol interacted with *E. coli* at MIC and 1/2MIC concentrations, the number of viable bacteria at the end of the 24 hours was calculated as $1.0x10^2$ and $2.7x10^6$ CFU/mL, respectively. When OEO interacted with MRSA at MIC and 1/2MIC concentrations, the number of viable bacteria at the end of the 24-hour was calculated as $8.6x10^1$ and $3.0x10^6$ CFU/mL, respectively. When carvacrol interacted with *E. coli* at MIC and 1/2MIC concentrations, the number of viable bacteria at the end of the 24 hours was calculated as $4.3x10^1$ and $1.5x10^6$ CFU/mL, respectively. In the control series, it was observed that the number of viable bacteria increased as the incubation time progressed.



(a) (b)

Figure 3. Plots of the mean values for the logarithm of colony forming units per milliliter versus time for *O. onites* essential oil and carvacrol tested on *E. coli* (a) and MRSA (b). The *O. onites* essential oil and carvacrol at MIC and 0.5xMIC concentrations were added at timepoint 0 and measurements were taken at 4-hour intervals for 24 hours. Each point represents the mean ± SD of three independent experiments.

3.5. Biofilm Assay

In this study, the effect of the OEO and carvacrol on biofilm formation was tested by the quantitative crystal violet method. The OEO and carvacrol were assessed for their ability to reduce the formation of bacteria biofilm using concentrations ranging from 1/2MIC, 1/4MIC, and 1/8MIC. The inhibitory effects of the OEO and carvacrol on biofilm formation in *E. coli* reached a statistically significant level at 25 µg/mL, and 12.5 µg/mL, respectively ($p \leq 0.0001$). The inhibitory effects of the OEO and carvacrol on biofilm formation in MRSA reached a statistically significant level at 6.25 µg/mL ($p \leq 0.001$ for OEO, $p \leq 0.0001$ for carvacrol). The formation of *E. coli* and MRSA biofilms was decreased with increasing the OEO and carvacrol concentrations (Figure 4 A, B).

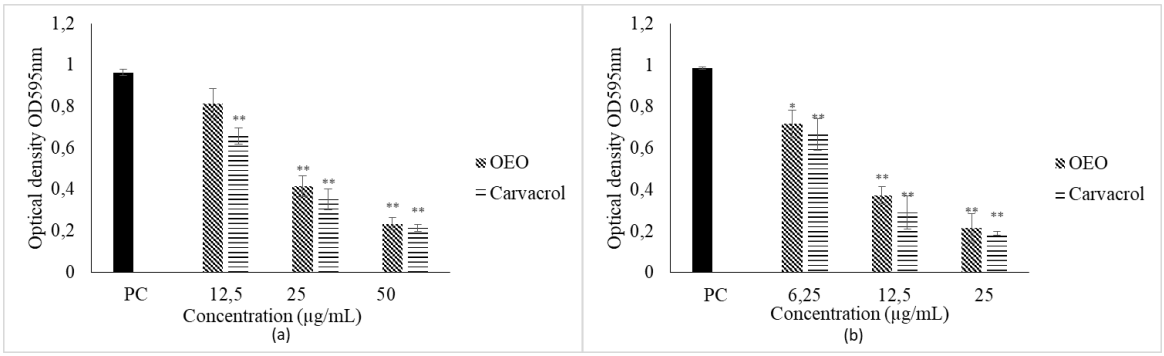


Figure 4. The effect of *O. onites* essential oil and carvacrol on biofilm formation in *E. coli* (a) and MRSA (b). PC, positive control (bacteria+medium). Each column represents the mean ± SD of three independent experiments. The asterisk indicates significant differences between the Optical Density (OD) values obtained against the different antibacterial agent concentrations evaluated and the control group (*$p \leq 0.001$, ** $p \leq 0.0001$).

The OEO inhibited *E. coli* biofilm formation at 50 µg/mL with 75.7% inhibition, followed by 25 and 12.5 µg/mL with 57% and 15.5% inhibition, respectively. Carvacrol inhibited *E. coli* biofilm formation at 50 µg/mL with 77.8% inhibition, followed by 25 and 12.5 µg/mL with 63.6% and 31.8% inhibition, respectively. The inhibition against MRSA biofilm formation was recorded for the OEO at 25 µg/mL with 78.3% inhibition, followed by 12.5 and 6.25 µg/mL with 62.1% and 27.3% inhibition, respectively. The carvacrol demonstrated inhibition of MRSA biofilm formation at 25 µg/mL with 80.7% inhibition, followed by 12.5 and 6.25 µg/mL with 70.6% and 32.4% inhibition, respectively (data not shown).

4. DISCUSSION

The chemical profile of the OEO was analyzed by GC-MS and a total of 44 components were characterized, representing 100% of the total chromatographic area. The most abundant phytochemical (53%) in the OEO was found to be carvacrol. The results of this study are compatible with the literature, as previous analyses have also concluded that carvacrol is typically the major constituent in the OEO. The carvacrol content in the oil ranges from approximately 50% to 85% [13, 14, 15]. The other main components identified in the OEO were linalool (13.05%), p-Cymene (12.64%), γ-Terpinene (3.37%), α-Pinene (1.82%), Caryophyllene (1.82%), α-Terpinene (1.54%), β-Myrcene (1.52%). These components have also been reported in other studies conducted with the OEO [13, 16, 17]. However, the levels of these components in essential oils have been found to vary. This variability is thought to be influenced by climatic and seasonal factors affecting the chemical composition and quantity of oils, as well as factors such as the type of plant, harvest period, geographical conditions, and distillation technique [18, 19].

In the disk diffusion test, the antibacterial activity of the OEO and carvacrol was investigated against *E. coli* and MRSA. The mean growth inhibition zones for the OEO against *E. coli* at concentrations of 0.25, 0.5, 1, 2, and 10 mg/mL were 22±1.4 mm, 30±0.4 mm, 38.7±1.4 mm, 45.8±1.8 mm, and 48.2±0.5 mm, respectively. The mean growth inhibition zones for the OEO against MRSA at concentrations of 0.25, 0.5, 1, 2, and 10 mg/mL were 22±1.6 mm, 34.2±0.6 mm, 41±1.2 mm, 47±1.6 mm, and 52.7±0.2 mm, respectively. The antibacterial activity of the OEO used in this study was higher than positive control at 0.5, 1, 2, and 10 mg/mL concentrations. It was observed that there was no statistically significant difference between the antibacterial activity of OEO against *E. coli* at a concentration of 0.25 mg/mL (22±1.4 mm) and the positive control ($p \geq 0.05$). In a previous study, ethanol extract obtained from *O. onites* was reported to show antibacterial activity with an inhibition zone diameter of 32.5 mm on *E. coli* at 1/50 concentration [20]. It was reported that the water extract obtained from *O. onites* formed an inhibition zone of 37 mm against *E. coli* ATCC25922 [21]. No study was found showing the antibacterial properties of OEO against MRSA used in this study.

The antibacterial activity of the carvacrol against *E. coli* at concentrations of 0.25, 0.5, 1, 2, and 10 mg/mL were 24±1.5 mm, 33.3±0.6 mm, 39.2±2.8 mm, 45.7±2.7 mm, and 49.9±2.4 mm, respectively. The antibacterial activity of the carvacrol against MRSA at concentrations of 0.25, 0.5, 1, 2, and 10 mg/mL were 23.3±1.1 mm, 35.5±1.1 mm, 42.3±0.9 mm, 48.2±1.1 mm, and 54.3±0.7 mm, respectively. The antimicrobial activity of the carvacrol used in this study was higher than positive control at 0.5, 1, 2, and 10 mg/mL concentrations. Similar to the results of this study, a previous study reported that 400 mg/mL carvacrol created a 35 mm inhibition zone against MRSA [22]. In a study, the mean growth of inhibition zones of carvacrol against *E. coli* at concentrations of 1000, 500, and 250 µg/mL were reported as 38 ± 5, 30 ± 4, and 18 ± 4 mm, respectively [23]. As a result of the disk diffusion test, it was determined that the inhibition zone diameters decreased in direct proportion to the decrease in the concentration of the OEO and carvacrol added to the test medium.

In the present study, the MIC value, which is the lowest OEO concentration that visually inhibits the growth of *E. coli*, was determined as 100 µg/mL. In addition, the MIC value for OEO was determined as 50 µg/mL against MRSA. The MBC value, which is the lowest essential oil concentration that kills 99.99% of bacteria, was determined as 200 µg/mL. In a study, the MIC value of *O. onites* methanol extract against *E. coli* ATCC11230 was reported as 1280 µg/mL [24]. In another study, the MIC value and the MBC value of oregano essential oil from Shanghai Sinopharm Chemical Reagent Co., Ltd (Shanghai, China) against MRSA were reported as 0.4 mg/mL [25]. In a study, it was found that the MIC and MBC of OEO against *S. aureus* were 0.125 and 0.25 mg/mL, respectively [26]. On the other hand, in the present study, MIC values of carvacrol against *E. coli* and MRSA were 100 and 50 µg/mL, respectively. This result was consistent with a previously reported study for the antibacterial properties

of carvacrol against *E. coli* (MIC of 100 µg/mL, and MBC of 100 µg/mL) [27]. In contrast, in another study, the MIC value of carvacrol against *E. coli* ATCC 25922 was reported as 256 µg/mL [28]. A previous study reported that carvacrol had a weak anti-staphylococcal activity against MRSA at a concentration of up to 1000 µg/disk [29]. However, another recent study reported that carvacrol had good anti-staphylococcal activity against MRSA with an MIC of 150 µg/mL [30]. This discrepancy may be due to the different techniques and concentrations used in these studies. The low MIC of carvacrol against Staphylococci was also supported by the present study. The results of these studies, together with the results in the current study, show that both carvacrol and OEO showed good antimicrobial activity against *E. coli* and MRSA. In addition, plant extracts are generally considered bactericidal if the MBC/MIC ratio is ≤4 and bacteriostatic if they are >4 [31]. In this study, the MBC/MIC values were determined as 2, so it can be said that OEO and carvacrol are bactericidal against *E. coli* and MRSA.

Antibacterial curve assay helps to understand the interactions that exist between antimicrobial agents and microbial strains. The assay indicates the time- or concentration-dependent effect of antimicrobial agents on microorganism species. It defines antimicrobial agents as bactericidal/fungicidal or bacteriostatic/fungistatic [32]. As a result of the antibacterial curve assay, it was observed that the bacteria remained viable from 0 to 24 h with gradually decreasing amounts. Bactericidal activity refers to a ≥3 $\log_{10}$ decrease in viability compared to the initial cultivation after 24 hours of exposure to the antimicrobial agent [33]. When OEO interacted with bacteria at MIC concentration, the number of viable *E. coli* and MRSA at the end of 24 hours was calculated as $2.9x10^2$ and $8.6x10^1$ CFU/mL, respectively. A decrease of 3 $\log_{10}$ CFU/mL were observed in *E. coli* and MRSA populations. Similarly, when carvacrol interacted with *E. coli* and MRSA at MIC concentration, the number of viable bacteria at the end of 24 hours was calculated as $1.0x10^2$ and $4.3x10^1$CFU/mL, respectively. A decrease of 3 $\log_{10}$ CFU/mL were observed in *E. coli* and MRSA populations. As a result, the bactericidal effects of OEO and carvacrol on *E. coli* and MRSA were confirmed using time-kill kinetics assay results. The present results were consistent with previous studies reporting that essential oils exhibited similar bactericidal activity with their phenolic compounds [34, 35].

Biofilm-associated infections are difficult to eradicate [36]. The use of antibiofilm agents that prevent bacteria from adhering to the surface may cause free-living cells to be easily attacked by the host immune system or antibiotic treatment [37]. Therefore, in recent years, the need for research focused on the discovery of new antibiofilm agents that prevent biofilm formation has increased. In the present study, the effects of OEO and carvacrol on biofilm formation of *E. coli* and MRSA were tested by the quantitative crystal violet method. The inhibitory effects of the OEO and carvacrol on the biofilm formation of *E. coli* reached a statistically significant level at 25 µg/mL, and 12.5 µg/mL, respectively ($p \leq 0.0001$). The inhibitory effects of the OEO and carvacrol on biofilm formation of MRSA reached a statistically significant level at 6.25 µg/mL ($p \leq 0.001$ for OEO, $p \leq 0.0001$ for carvacrol). The formation of *E. coli* and MRSA biofilms was decreased with increasing OEO and carvacrol concentrations.

OEO inhibited the biofilm formation in *E. coli* by 75.7%, 57%, and 15.5% at 1/2MIC, 1/4MIC, and 1/8MIC concentrations, respectively. In another study conducted with *E. coli* (Strain no: 97010), it was observed that commercially purchased *O. onites* essential oil reduced biofilm formation at subinhibitory concentrations. MIC and 1/2MIC doses have been reported to show a greater effect than 1/4MIC. It has been reported that a statistically significant decrease was observed even at the MIC level in biofilms treated with *O. onites* essential oil [38]. It has been reported oregano oil and carvacrol at sub-inhibitory concentrations (<0.01%, which is 0.2xMIC) showed antibiofilm activity against uropathogenic *E. coli* [39]. Carvacrol inhibited *E. coli* biofilm formation at 50 µg/mL with 77.8% inhibition, followed by 25 and 12.5 µg/mL with 63.6% and 31.8% inhibition, respectively. The inhibition against MRSA biofilm formation was recorded for the OEO at 25 µg/mL with 78.3% inhibition, followed by 12.5 and 6.25 µg/mL with 62.1% and 27.3% inhibition, respectively. The carvacrol demonstrated inhibition of MRSA biofilm formation at 25 µg/mL with 80.7% inhibition, followed by 12.5 and 6.25 µg/mL with 70.6% and 32.4% inhibition, respectively. In previous studies, it has been reported that the MIC value of

carvacrol against MRSA is 150 µg/mL and that 75 µg/mL of carvacrol inhibits biofilm formation by 93% [30]. In the presence of carvacrol (1/2MIC), the mean biofilm formation value for *S. aureus* were reported to be equal to 28.3% [40].

In this study, it was shown that OEO and carvacrol interfere with biofilm formation during planktonic growth. The reasons for this may be due to multiple factors acting synergistically or individually. The antimicrobial activity of oregano oil is mostly attributed to the action of its main phenolic component, carvacrol, which showed significant bactericidal activity when tested separately [41]. On the other hand, carvacrol interacts with the lipid bilayer of the cytoplasmic membrane due to its hydrophobic structure, causing loss of integrity and leakage of cellular materials such as nucleic acids, ATP, and ions [42].

Essential oils tend to be more potent compared to isolated compounds due to their ability to work synergistically, affecting multiple targets simultaneously, unlike pure compounds that typically have a singular mode of action. This multi-targeted approach can enhance the overall effectiveness of essential oils [43]. Other studies have also reported that essential oils rich in carvacrol, such as *O. onites*, have significant antimicrobial activity [44, 45]. Carvacrol, which has a monoterpene structure, is a phenolic compound found in essential oils. It can exhibit hydrophobic properties due to the aromatic chain in its structure and hydrophilic properties due to the phenolic -OH group. These properties in the chemical structure of carvacrol are considered to be the reason for its antimicrobial activity [46]. Carvacrol has also been shown to attack the external membrane of Gram-negative bacteria [47]. However, its main area of action is stated to be the cytoplasmic membrane, causing the transport of ions across the membrane without consuming energy. Cells exposed to carvacrol are thought to change the fatty acid composition of the cell membrane as an adaptation mechanism to maintain membrane structure and function [48, 49]. The use of phenolic compounds is suggested due to the high antimicrobial activity and the variance of phenolic composition in essential oils originating from different geographical locations. However, more researches are needed to determine the effective dosage and various mechanisms of action of OEO and carvacrol for clinical trials. In the light of these results, OEO and carvacrol can be new therapeutic agents for *E. coli* and MRSA.

5. CONCLUSION

One of the alternative strategies suggested to overcome the resistance problem is the use of plant essential oils with antimicrobial properties and the substances found in them. The OEO and carvacrol may be an effective source of natural supplements to treat infections caused by MRSA and *E. coli* bacteria. The findings also suggest that OEO and carvacrol can be used to treat diseases caused by *E. coli* and MRSA without allowing the development of resistance. However, *in vivo* antibacterial evaluation using experimental animals, toxicity tests, and pharmacokinetic properties of the active components (especially carvacrol) need to be investigated.

ACKNOWLEDGMENTS

No source of financial support was received for his study. I thank ‘Innovative Technologies Application and Research Center (YETEM)’ in Süleyman Demirel University, Isparta/Turkey, for expert cooperation in performing the GC-MS analysis.

CONFLICT OF INTEREST

The author stated that there are no conflicts of interest regarding the publication of this article.

CRediT AUTHOR STATEMENT

Demet Hançer Aydemir: Conceptualization, Investigation, Formal analysis, Writing – Original Draft, Visualization.

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ESKİŞEHİR TEKNİK ÜNİVERSİTESİ BİLİM VE TEKNOLOJİ DERGİSİ
C- YAŞAM BİLİMLERİ VE BİYOTEKNOLOJİ

Eskişehir Technical University Journal of Science and Technology
C -Life Sciences and Biotechnology

Estuscience – Life, 2025, 14 (2) pp. 59 -65, DOI: 10.18036/estubtdc.1609497

RESECRH ARTICLE

FENTANYL GENOTOXICITY EVALUATION VIA COMET ASSAY

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Abstract

DNA is our main genetic structure that can be affected by various molecules we come into contact with. Therefore, there is a high probability of DNA damage. Genotoxicity tests involve systematic methods designed to assess the safety of drugs and chemicals. Comet assay evaluates the possible genotoxicity resulting from DNA single-strand breaks.

Fentanyl is a powerful painkiller from the group called opioid analgesics administered intravenously. In this research, we evaluated the possible DNA damage in human lymphocytes after fentanyl exposure via single-cell gel electrophoresis (Comet Assay).

DNA damage in healthy human peripheral lymphocytes treated with fentanyl was investigated via comet assay. Lymphocytes were treated with 5, 10, 20, and 40 µg/mL doses of fentanyl for 1 hour. After the incubation period, the cells' DNA tail length, tail intensity, and tail moment values were evaluated by comparing them with the spontaneous control and positive control data. EtBr stained slides were visualized under the fluorescent microscope. Fentanyl induced the comet parameters such as tail length, tail intensity, and tail moment but dose-dependent increase was not obtained after fentanyl administration. Fentanyl showed the highest tail length and tail moment value at the dose of 10 µg/mL. The highest tail intensity value was obtained at the dose of 40 µg/mL fentanyl administration.

This study aimed to reveal previously undiscovered genotoxicity of fentanyl on healthy human lymphocytes in vitro via comet assay. Fentanyl exposure induces DNA damage in healthy human lymphocytes, as shown via comet parameters (tail length, tail intensity, and tail moment). Genotoxic effect does not display a consistent dose-dependent increase.

Keywords

Comet assay,
Single-cell gel electrophoresis,
DNA damage,
Lymphocyte culture,
Genotoxicity,
Fentanyl

Time Scale of Article

Received :29 December 2024
Accepted : 15 February 2025
Online date : 25 July 2025

1. INTRODUCTION

Genotoxic effects of anesthetic agents are very important research area [1,2]. Applications for determining genotoxic damage in patients due to frequent exposure to anesthetics are important. Further studies with comet assay is necessary to clarify and follow up the extent of genotoxic damage.

In this research, possible genotoxic effects of fentanyl was evaluated in human lymphocytes via comet assay. Fentanyl is a powerful rapid-acting painkiller, originally developed in 1960 by Dr. Paul Janssen [3]. Because of its potency, effectiveness, and rapid onset of action, Fentanyl is one of the most widely

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used opioids and is commonly used to manage acute and chronic pain. Fentanyl is approximately 50-100 times more potent than the known natural opioid morphine [4,5].

Lymphocytes were chosen for the comet assay due to their high sensitivity to DNA damage, reflecting systemic exposure to genotoxic agents. Their non-invasive sampling makes them ideal for assessing whole-body effects. Given fentanyl's impact on multiple organ systems, evaluating its genotoxicity in lymphocytes provides insight into potential systemic toxicity [6].

Comet assay has been shown to be a highly sensitive, rapid, visual and rational method for the evaluation of DNA single strand breaks in individual cells [7, 8]. The effects of opioids, such as fentanyl, on immune function are a critical issue that highlights the importance of dosage. In a clinical study, the doses of fentanyl administered to healthy individuals were examined. Participants received an initial dose of fentanyl of 3 micrograms/kg via IV, followed by a 2-hour IV infusion at a dose of 1.2 micrograms x kg (-1) x h (-1). Various tests were conducted to evaluate immune function before and after fentanyl administration. The analysis revealed that fentanyl did not suppress immune resistance; in fact, it increased natural killer cell cytotoxicity. Results suggest that careful consideration of dosage is essential for understanding the effects of opioids, such as fentanyl, on immune system [9].

It is very important to determine DNA damage in patients, especially in repeated anesthesia applications, and it is very important to reveal genotoxicity with the comet assay to clarify the degree of damage that may occur in case of frequent exposure to anesthetics such as fentanyl [10, 7].

Researches on genotoxic of fentanyl is limited. Genotoxicity of fentanyl has been investigated and results were reported with bacterial mutation assay [11]. To our knowledge, no such studies have been conducted with fentanyl in vitro comet assay by using human lymphocytes [12].

As understood from these information, genotoxicity of fentanyl data was limited so 5, 10, 20, and 40 µg/mLof fentanyl concentrations were applied on human lymphocyte cultures in vitro.

2. METHODS

The aim of the comet assay is to achieve at least 80% viability in cells exposed to an agent. Based on this result, fentanyl concentrations of 5, 10, 20, and 40 µg/mL were used for the comet experiments.

The peripheral blood samples were then transferred to tubes containing Histopaque and centrifuged at room temperature and centrifuged 400 rcf for 30 minutes. Lymphocytes separated due to the density difference after centrifugation are added to the lymphocytes in these falcons with 6 mL of PBS solution washed with PBS solution. The separated lymphocytes were transferred to separate eppendorf tubes and Trypan blue staining was used to confirm viability above 80% in isolated lymphocytes. The samples were subsequently placed in remifentanil concentrations and incubated for an hour at 37°C. At the end of the incubation period, eppendorf tubes were centrifuged at 300 rpm for 5 minutes, the supernatant was removed. Next, 100 µL of a low-melting-point agar, mixed with the 100 µL lymphocyte solution, was dripped onto slides previously coated with high-melting-point agar. These slides were then covered with a 24 x 60 mm coverslip. After incubating the slides in the refrigerator for 15-20 minutes, the coverslips were separated from the slides and placed in chalets containing a lysis solution. They were kept in the refrigerator for 1 hour. Following lysis, the slides were placed in horizontal tanks containing an electrophoresis buffer solution with a pH greater than 13 for 20 minutes to denature the DNA. Electrophoresis was conducted at 25 V and 300 mA in running buffer [13]. Following electrophoresis, the slides neutralized with a neutralizing buffer (pH 7.5) for 5 minutes [14]. Subsequently, 20 µL of a 20 µg/mL ethidium bromide (EtBr) solution was evenly applied over the slides, which were then covered with a 24 x 60 mm coverslip. All procedures were performed in a darkened area to prevent DNA damage. For comet analysis, the slides were examined at 40x magnification using a fluorescent microscope. Image analysis and comet counting were conducted on 100 comets [15]. Fluorescent EtBr staining was

utilized to detect damage, and images acquired under a fluorescent microscope were analyzed using the Comet Image Processing and Analysis System Software (Comet IV).

2.1. Statistical Analysis

The comet parameters of fentanyl was investigated in 100 cells for each dose of fentanyl, and then statistical analysis was performed by comparing them with the positive and negative control data. Structural changes in tail lengths, tail moments and tail densities were assessed by comparing the number of comets in the cells with the negative and positive controls. These parameters entered into Graphpad Prism 8 program and analyzed separately. Since the variance values between groups in the parameters were different, the Dunnet T3 test was chosen and comparisons were made with the control groups.

3. RESULTS

The clinical relevance of fentanyl's genotoxicity related to its potential to cause genetic damage, which could lead to mutations, cancer, or other genetic disorders. Understanding this aspect is crucial for assessing the long-term risks associated with fentanyl exposure, especially given its widespread medical use and potential for abuse. Currently, direct studies on fentanyl's genotoxic effects are limited. However, research on certain fentanyl analogues provides some insights. A study titled "The Genotoxicity of Acrylfentanyl, Ocfentanyl and Furanylfentanyl" assessed the genotoxic potential of these compounds. The findings indicated that these analogues could induce genetic damage, suggesting a possible genotoxic risk [5]. The aim of the current research is to investigate the potential genotoxicity of fentanyl using the via in vitro comet assay in cultured healthy human lymphocytes, which is worldwide well known genotoxicity test [16, 17, 18, 19, 20].

DNA damage in healhty human peripheral lymphocytes treated with fentanyl was investigated via comet assay. Doses within the range of IC50 or LD50 dose values were used in the comet assay. Lymphocytes treated with 5, 10, 20, and 40 µg/mL doses of fentanyl for 1 hour. After the incubation period the DNA tail length, tail intensity, and tail moment values of the cells were evaluated by comparing them with the spontaneous control and positive control data. EtBr staining was performed and slides were visualised under flourescent microscobe (Figure 1). Fentanyl induced the comet parameters as shown in Figure 2, 3, 4. Fentanyl showed the highest tail length and tail moment value at the dose of 10 µg/mL (Figure 2, 4). The highest tail intensity value was obtained at the dose of 40 µg/mL fentanyl administration (Figure 3).

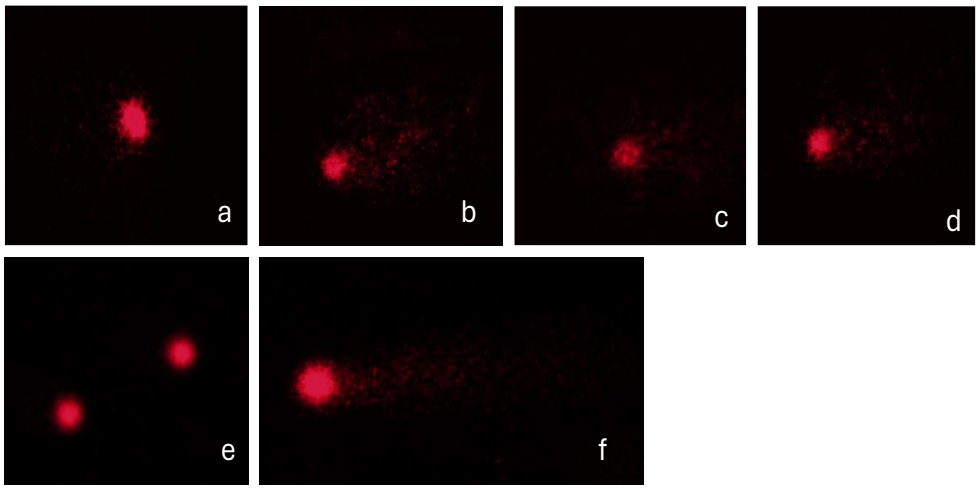

Figure 1. Human peripheral lymphocyte cells treated for 1h with (a) 5 µg/mL (b) 10 µg/mL, (c) 20 µg/mL, (d) 40 µg/mL dose of fentanyl (under 40X) (e) The negative control exhibits intact nuclei with minimal DNA damage, while the positive control which is (f) 50 µM H_2O_2 displays significant DNA fragmentation, characteristic of extensive DNA damage.

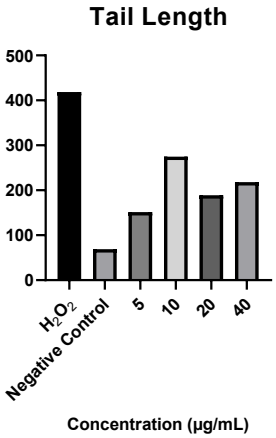

Figure 2. Tail length of DNA in peripheral lymphocyte cells treated for 1h with doses of fentanyl. There is a dose-dependent increase in tail length from 5 to 10 µg/mL. H2O2 as a Positive Control, the highest tail length (~450 µm), indicating significant DNA damage. Negative Control, minimal tail length, as expected for an untreated control.

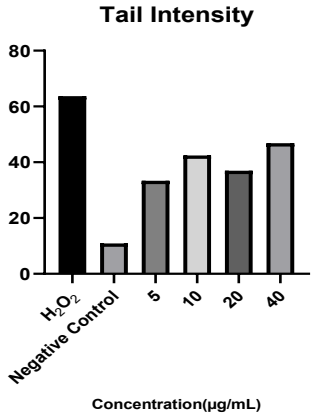

Figure 3. Tail intensity of DNA in peripheral lymphocyte cells treated for 1h with doses of fentanyl. Dose-dependent increase in tail intensity (except 20µg/ml). H2O2 as a Positive Control, the highest tail length (~450 µm), indicating significant DNA damage. Negative Control, minimal tail length, as expected for untreated control.

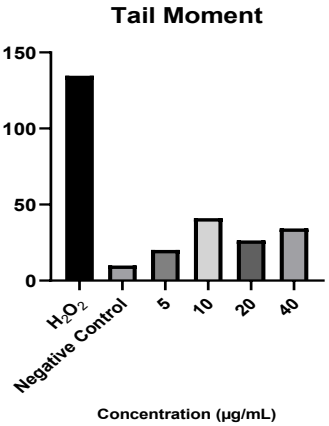

Figure 4. Tail moment of DNA in peripheral lymphocyte cells treated for 1h with doses of fentanyl. H2O2 as a Positive Control, the highest tail length (~450 µm), indicating significant DNA damage. Negative Control, minimal tail length, as expected for untreated control.

Table 1. DNA damage in peripheral lymphocyte cells treated with doses of fentanyl for 1 hour.

	Treatment	**Tail Length**	**Tail Intensity**	**Tail Moment**
	Concentraion (µg/mL)	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**
Negative Control	-	69,00±4,629	10,93±2,221	9,91±4,177
H_2O_2(Positive Control)	50 µM	414,9±12,71***	63,71±4,864***	134,7±11,49***
Fentanyl	5 µg/mL	151,6±63,54***	33,35±23,74***	20,23±18,42**
	10 µg/mL	274,7±119,5***	42,45±22,85***	41,12±31,07***
	20 µg/mL	189,1±99,21	36,96±26,65	26,42±22,98
	40 µg/mL	218,0±71,30***	46,83±25,87***	34,27±27,14***

(Table 1 descriptions) H_2O_2: Hydrogen Peroxide (Positive Control), ±SD: Standard Deviation Values, (Dunnet T3Test), ** P≤ 0.05 *** P≤ 0.001 gives significance values relative to control groups.

Table 1 presents the mean ± standard deviation (SD) of tail length, tail intensity, and tail moment in the comet assay for different treatment groups. The negative control represents untreated cells, while H_2O_2 (50 µM) serves as the positive control to induce significant DNA damage. The statistical significance of differences between treated groups and the negative control was analyzed using Dunnett's T3 test. H2O2: Hydrogen Peroxide (Positive Control), ±SD: Standard Deviation Values, (Dunnet T3Test), ** P≤ 0.05 *** P≤ 0.001 gives significance values relative to control groups.

The comet parameters for fentanyl was investigated in 100 cells for each dose of fentanyl, and then statistical analyze was performed by comparing them with the positive and negative control data. Negative control indicates minimal DNA damage. The positive control (H_2O_2) shows significantly higher comet parameters compared to the negative control. This is consistent with DNA damage caused by oxidative stress and confirms the sensitivity of the test. At the dose of 5 µg/mL, DNA damage increases compared to the negative control, as shown by the comet parameters. Statistical significance indicates that this concentration (10 µg/mL) has a genotoxic effect. DNA damage becomes more evident at the dose of 10 µg/mL, with significant increases in all comet parameters compared to the negative control. This suggests a dose-dependent response. Values of 20 µg/mL dose are slightly lower than 10 µg/mL, although still high compared to the negative control. This introduces variability or non-linear dose response. DNA damage in cells exposed to fentanyl was significantly higher than in the negative control but lower than the positive control (H_2O_2) (Table 1). These parameters entered into Graphpad Prism 8 program and analyzed separately. Since the variance values between groups in the parameters were different, the Dunnet T3 test was chosen and comparisons were made with the control groups.

4. DISCUSSION

Since DNA can be damaged by various chemicals through different mechanisms, it is important reveal the genotoxicity of agents encountered by human via different genotoxicity test systems. In conclusion, under the conditions used in this study (short-term exposure), results indicate that evaluating the genotoxicity of fentanyl maintained anesthesia compared to the positive control is important,

considering dose and continuous exposure. It is crucial to conduct comet assay experiments with blood samples from patient profiles regularly exposed to long-term fentanyl exposure.

Fentanyl exposure induces DNA damage in healthy human lymphocytes, as shown via comet parameters. However, the genotoxic effect does not display a clear dose-dependent manner, suggesting that fentanyl may cause DNA damage through mechanisms that are not linearly dose-dependent manner. This effect at 20 µg/mL dose revealed nonlinear comet parameters. This dose response may suggest potential thresholds or cellular adaptation mechanisms.

Future research should focus on the specific pathways through fentanyl causes DNA damage. Additionally, it will be necessary to investigate in vivo whether the effects of fentanyl on DNA are reversible. In vivo evaluation of the long-term effects of repeated or chronic fentanyl exposure on DNA integrity would be very informative.

ACKNOWLEDGEMENTS

Authors of the manuscript wish to thank Prof. Dr. Berrin AYAZ TUYLU.

FUNDING

Not applicable

CONFLICT OF INTEREST

Authors of the manuscript have no conflict of interest regarding the publication of the manuscript.

CRediT AUTHOR STATEMENT

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CONSENT OF THE PUBLICATION

Oge Artagan and Bahar Koklu agreed to publish the manuscript.

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ESKİŞEHİR TEKNİK ÜNİVERSİTESİ BİLİM VE TEKNOLOJİ DERGİSİ
C- YAŞAM BİLİMLERİ VE BİYOTEKNOLOJİ

Eskişehir Technical University Journal of Science and Technology
C -Life Sciences and Biotechnology

Estuscience – Life, 2025, 14 (2) pp. 66-72, DOI: 10.18036/estubtdc.1703277

RESEARCH ARTICLE

11β-HSD1 REGULATES GLUT1 EXPRESSION IN HUMAN BRAIN MICROVASCULAR ENDOTHELIAL CELLS

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Abstract

11β-Hydroxysteroid dehydrogenase type 1 (11β-HSD1) locally regenerates active glucocorticoids and has been linked to metabolic dysfunction and neurodegeneration. In this study, we aimed to investigate whether 11β-HSD1, an enzyme known to modulate glucocorticoid activity and metabolic homeostasis, plays a regulatory role in the expression of glucose transporter-1 (GLUT1) in human brain microvascular endothelial cells (HBEC5-i), which are critical for maintaining blood–brain barrier integrity and cerebral energy balance. HBEC5-i were transduced with a GFP-tagged pLKO.1 lentiviral vector (MOI 10) encoding an shRNA against 11β-HSD1 or with a non-targeting control. Transduction efficiency was confirmed by GFP fluorescence and knockdown was validated by immunoblotting. Protein abundance of GLUT1, CPT1A, PFKFB3 and GSK3α/β was quantified by western blotting. 11β-HSD1 knockdown reduced GLUT1 and GSK3α/β while CPT1A and PFKFB3 remained unchanged. While the association between 11β-HSD1 and energy metabolism is well-documented, the precise molecular mechanisms governing this relationship remain incompletely understood. Our study is the first to explore this interaction specifically in HBEC5-i, providing foundational insights that not only elucidate the metabolic roles of 11β-HSD1 in this unique cellular context but also pave the way for future research aimed at uncovering the downstream signaling pathways and therapeutic potential of targeting 11β-HSD1 in cerebrovascular disorders.

Keywords

11β-HSD1,
GLUT1,
Brain microvascular endothelial cells,
Energy metabolism,
Blood–brain barrier

Time Scale of Article

Received :21 May 2025
Accepted : 04 July 2025
Online date :25 July 2025

1. INTRODUCTION

The endothelium is a complex and diverse organ in the human body, consisting of multiple subtypes of endothelial cells that differ in their characteristics, function, and location. These endothelial cells can be categorized into arterial, venous, microvascular, and lymphatic types, each with different roles and varying oxygen levels [1]. For example, pulmonary microvascular endothelial cells differ significantly from pulmonary arterial endothelial cells in their oxygen and glucose consumption and intracellular ATP levels [2]. This difference in metabolic activity may be attributed to the different oxygen levels in their respective environments. Furthermore, brain microvascular endothelial cells possess more mitochondria than peripheral endothelial cells, which suggests an increased oxidative metabolism in these cells [3]. However, more research is needed to confirm this hypothesis. Overall, the endothelium is a highly heterogeneous and complex organ, and understanding the differences between its various subtypes is essential for developing treatments for diseases and disorders that affect the endothelium.

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Endothelial cells, cells lining the inner surface of blood vessels, play a crucial role in maintaining vascular homeostasis, which is critical for proper brain function. These cells provide 90% of their energy through glycolysis under normal conditions. However, recent studies have shown that quiescent endothelial cells perform FAO in basal metabolism to maintain tight junctions, redox homeostasis, and glucose transport [4]. This function is paramount in ensuring brain health and energy homeostasis, particularly in association with neurodegenerative diseases related to aging and diet. Detailed metabolic studies have revealed that endothelial cells require high glycolysis and FAO during angiogenesis, forming new blood vessels from pre-existing ones. During angiogenesis, endothelial cells switch from hypometabolism to hypermetabolism, which is essential for understanding the metabolic changes in the vascular structure that deteriorate with aging and pathophysiological neurodegenerative diseases such as TBI (traumatic brain injury) and CTE (chronic traumatic encephalopathy).

11β-Hydroxysteroid dehydrogenase type 1 (11β-HSD1) is an NADPH-dependent enzyme that catalyzes the conversion of inactive glucocorticoids (cortisone in humans) to their active forms (cortisol), exerting significant influence over local glucocorticoid availability within tissues [5],[6]. This enzyme is broadly expressed across various tissues, including the liver, adipose tissue, skeletal muscle, and the central nervous system, where it modulates glucose metabolism, inflammation, and cellular stress responses [7], [8]. In endothelial cells, which line the interior surface of blood vessels, 11β-HSD1 has been implicated in regulating vascular homeostasis and metabolic functions. Recent studies have highlighted that 11β-HSD1 may influence endothelial barrier integrity, reactive oxygen species (ROS) production, and endothelial cell proliferation [9]. Although the impact of 11β-HSD1 on metabolic pathways has been well-characterized in other cell types, its specific role in brain endothelial cells, which maintain the critical blood-brain barrier (BBB), remains largely unexplored. Understanding how 11β-HSD1 modulates glucose transport and metabolic signaling in these cells is essential for elucidating its contribution to cerebrovascular health and disease.

Brossaud, et al., (2023) revealed that 11β-HSD1 inhibition prevented hippocampal-related memory deficiency in diabetic juvenile rats [8]. In a study conducted with PET scan analysis, BMI (from lean to obese) and age factors measured 11β-HSD1 expression in the brain. The increase in BMI index and aging shows that 11β-HSD1 expression increases in the brain. Excessive amounts of cortisol in persistency CSF were detected in the first week after TBI, and researchers reported that this may be due to BBB disruption. In addition, cortisol, a glucocorticoid steroid hormone, is primarily responsible for stimulating gluconeogenesis in the liver and promoting adipocyte differentiation and maturation. Prolonged excess cortisol leads to visceral adiposity, insulin resistance, hyperglycemia, memory dysfunction, cognition impairment, and more severe Alzheimer's Disease [9].

We experimented with investigating the response of HBEC5-i when we silenced the expression of the 11β-HSD1 enzyme using pLKO lentivirus. After collecting the cells, we examined the protein expression of the resistant cells obtained after the puromycin selection process. Although the remaining cells did not proliferate, they survived for ten days. We investigated the changes in protein expressions that occurred due to suppressing 11β-HSD1 expression. Our study revealed that the expression of GLUT1, GSK3α and GSK3β in HBEC5-i's was significantly reduced. However, there was no significant change in the expressions of CTP1A and PFKFB3. These results suggest that 11β-HSD1 may regulate glycolysis and glycogen synthesis, but we need to clarify its mechanism further.

2. MATERIALS AND METHODS

2.1. Human Brain Microvascular Endothelial Cell (HBEC-5i) Culture

Human brain microvascular endothelial cells (HBEC-5i; ATCC® CRL-3245™), derived from male donor brain microvessels and immortalized via SV40 large T antigen, were cultured in T75 flasks pre-

coated with 0.2% (w/v) gelatin (porcine Type A; Sigma-Aldrich). Cells were maintained in a 1:1 mixture of endothelial cell growth medium (VEC Technologies, Rensselaer, NY, USA) and Medium 199 (Corning, Manassas, VA, USA). The base medium included 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Invitrogen) unless otherwise specified. Cultures were incubated at 37 °C in a humidified 5% CO_2 atmosphere and passaged upon reaching 80–90% confluence using standard trypsinization protocols. For in vitro treatment studies, HBEC-5i cells were seeded into gelatin-coated multi-well plates.

2.2. Silencing of 11β-HSD1 expression by lentiviral vector in HBEC5-i

The lentivirus pLKO.1-puro-CMV-tGFP (from Sigma Aldrich's MISSION) with Clone ID TRCN0000028065 and sequence 'CGAGCTATAATATGGACAGAT' was used to silence 11B-HSD1 expression in BMVECs. The transfection was done using polybrene. The cells were seeded 24 hours prior to transfection in a 24 well plate, with 6-8x10^{-4} cells per well. Various groups were established, including negative control (uninfected cells), positive control (GFP-lentivirus (MISSION, pLKO.1-puro Control), Blank and lentivirus injection) to monitor puromycin selection. We made sure to observe 30-40% confluency during the process. The multiplicity of infection (MOI), defined as the number of virus particles per cell, was calculated as 10 for lentiviral injection.

2.3. Western Blotting

Equal amounts of total protein (9 µg per lane) were resolved by SDS-PAGE using 4–12% Bis-Tris polyacrylamide gels and subsequently transferred to nitrocellulose membranes. Membranes were blocked for 1 hour at room temperature in 5% nonfat dry milk diluted in Tris-buffered saline with 0.1% Tween-20 (TBST), followed by overnight incubation at 4 °C with primary antibodies diluted in 5% bovine serum albumin (BSA) in TBST. Target antibodies were used at a 1:2,000 dilution; β-actin (loading control) was incubated separately at a 1:10,000 dilution. All antibodies purchased from Cell Signaling company except 11β-HSD1 antibody. Codes represents antibodies product number of cell signaling company. GSK3α-β (D5C5Z), PFKFB3 (13123), CPT1A (D3B3), 11β-HSD1 (NBP1-32027, Novus). Following primary antibody incubation, membranes were washed with TBST and probed for 1 hour at room temperature with species-specific horseradish peroxidase (HRP)–conjugated secondary antibodies (1:5,000 dilution). Prestained molecular weight markers were run in parallel to confirm protein band sizes. Signal detection was performed using enhanced chemiluminescence (ECL) reagents, and membranes were imaged using the Amersham Imager 680 system (GE Healthcare Biosciences, Marlborough, MA, USA). Relative protein expressions were quantified by densitometric analysis in ImageJ software and normalized to β-actin expression for each lane.

2.4. Statistics

All data were analyzed using GraphPad Prism (v10.0; GraphPad Software, San Diego, CA, USA). For multi-group comparisons, one-way ANOVA with appropriate post hoc corrections (Tukey's) were used.

3. RESULTS

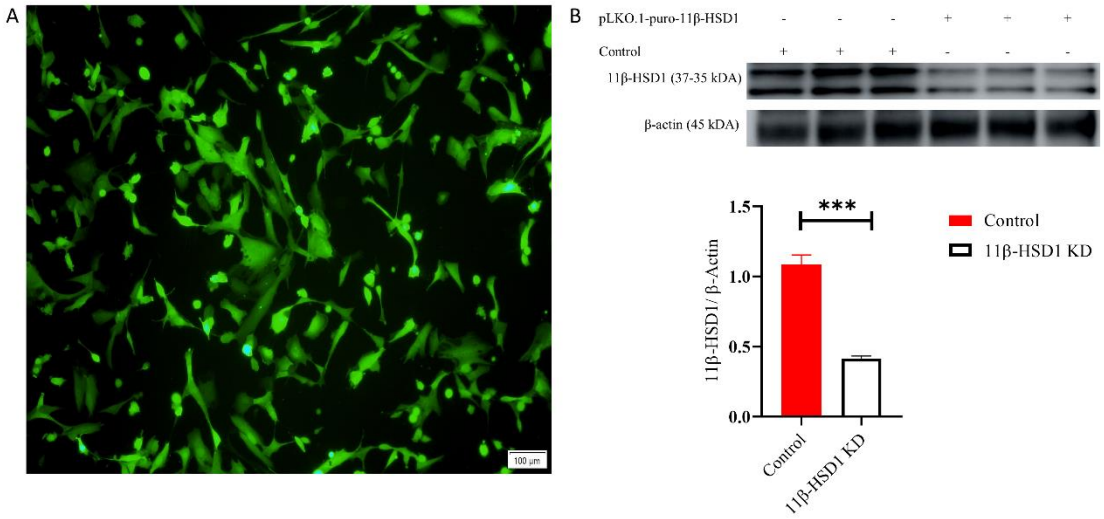

Figure 1. Efficient 11β-HSD1 knock-down in HBEC5-i cells.

GFP fluorescence imaging showing successful lentiviral transduction of HBEC5-i cells with pLKO.1-puro-CMV-tGFP-11β-HSD1 construct. Puromycin selection (2 µg/mL) was applied for 10 days. (Figure 1A) Western blot analysis confirming the efficient knockdown (KD) of 11β-HSD1 protein expression in HBEC5-i cells. β-Actin served as the control. Data are representative of three independent experiments (Figure 1B) (*p<0.05).

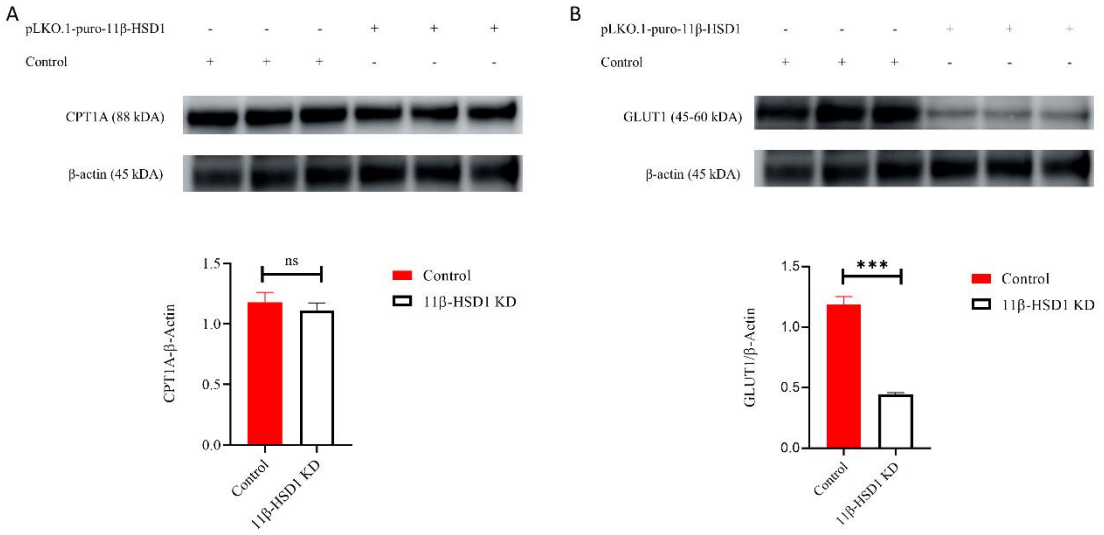

Figure 2.11β-HSD1 Knockdown reduces GLUT1 expression but not change CPT1A expression in HBEC5-i Cells.

Western blot analysis demonstrated that knockdown of 11β-HSD1 in HBEC5-i cells resulted in a significant reduction in GLUT1 protein levels compared to the control group (p<0.001) (Figure 1B). As

GLUT1 is the principal glucose transporter at the blood-brain barrier, its downregulation indicates impaired glucose uptake capacity in endothelial cells.

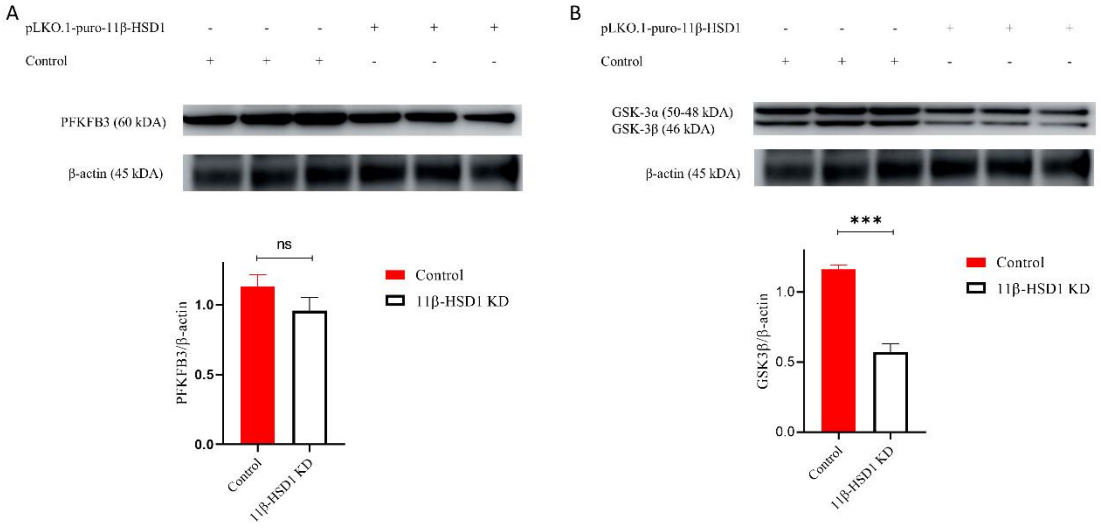

Figure 3. GSK-3α and GSK-3β expression is reduced following 11β-HSD1 knockdown. The expression level of PFKFB3 remains unchanged.

Further analysis revealed that 11β-HSD1 silencing led to a significant reduction in GSK3β protein expression ($p<0.01$), while GSK3α expression showed a decreasing trend but did not reach statistical significance ($p>0.05$) (Figure 3B). Notably, the reduction in GSK3β expression was more pronounced compared to GSK3α (Figure 3B). Notably, the decrease in GSK3β expression was more pronounced compared to GSK3α. Given the roles GSK3 in regulating glycogen synthesis and cell survival pathways, these changes suggest metabolic alterations associated with impaired glucose handling in endothelial cells. No significant differences were observed in PFKFB3 and CPT1A expression levels between 11β-HSD1 knockdown cells and controls ($p>0.05$) (Figure 1A and Figure 3A). This finding shows that protein-level expression of key regulators of fatty-acid oxidation and glycolysis remained largely unchanged, implying that 11β-HSD1 exerts a targeted influence on glucose transport rather than broadly reshaping cellular energy metabolism.

4. DISCUSSION

The findings from this study demonstrate that the suppression of 11β-HSD1 expression significantly alters the metabolic profile of human brain microvascular endothelial cells (HBEC5-i). Most notably, the downregulation of GLUT1, a key glucose transporter in endothelial cells, highlights a potential regulatory role of 11β-HSD1 in glucose uptake and cellular energy metabolism[10]. GLUT1 is essential for maintaining cerebral glucose homeostasis, and its decreased expression in 11β-HSD1 knockdown (KD) cells suggests that 11β-HSD1 positively influences glucose transport in brain endothelial cells. This supports the hypothesis that 11β-HSD1 may modulate glycolytic flux via regulation of GLUT1 expression, especially under conditions requiring increased energy demand such as neuroinflammation or injury.

The reduction of GSK3α and GSK3β further suggests a broader role for 11β-HSD1 in metabolic regulation. GSK3 is known to regulate glycogen synthesis and multiple signaling pathways related to cellular survival and inflammation [11]. The more pronounced decrease in GSK3β expression may

indicate isoform-specific sensitivity to metabolic stress in HBEC5-i. Interestingly, no significant changes were observed in CPT1A or PFKFB3 expression. CPT1A, a key enzyme in fatty acid oxidation, and PFKFB3, a regulator of glycolysis, are typically involved in endothelial cell metabolism during angiogenic activity [12]. Their unchanged levels imply that 11β-HSD1 might primarily impact glucose transport mechanisms rather than directly modulating glycolysis or fatty acid oxidation pathways at the transcriptional or translational level.

The survival of 11β-HSD1 KD cells, despite reduced proliferative capacity, indicates that metabolic adaptation mechanisms may partially compensate for the loss of 11β-HSD1 activity. These cells might rely on alternative energy pathways or residual glycolytic activity for maintenance. Our findings align with previous reports implicating 11β-HSD1 in metabolic disorders, cognitive decline, and neurodegeneration. Elevated cortisol levels, regulated by 11β-HSD1, have been associated with blood-brain barrier dysfunction and increased neuroinflammatory signaling [9]. Thus, the observed metabolic alterations in endothelial cells may represent an early molecular event in the pathogenesis of neurological diseases, such as Alzheimer's disease and traumatic brain injury [8],[13].

Limitations of this study include the lack of transcriptomic validation and functional assays such as glucose uptake measurements or cell viability tests post-GLUT1 downregulation. Additionally, mechanistic insights into how 11β-HSD1 regulates GLUT1 at the molecular level remain to be elucidated. Future studies should aim to clarify the signaling cascades involved, explore the interplay with other metabolic regulators, and assess the *in vivo* relevance of our findings. Investigating whether pharmacological inhibition of 11β-HSD1 alters blood-brain barrier integrity and glucose transport in animal models could provide translational insights.

In summary, our study identifies 11β-HSD1 as a regulator of GLUT1 expression in human brain microvascular endothelial cells, implicating this enzyme as a key metabolic modulator in the cerebrovascular system.

CONFLICT OF INTERESTS

The authors stated that there are no conflicts of interest regarding the publication of this article.

ACKNOWLEDGEMENTS

This study was supported by the TÜBİTAK BİDEB 2214-A International Research Fellowship Program. With the help of this fellowship, I was able to utilize the research infrastructure of the Medical University of South Carolina (MUSC). I would like to express my sincere gratitude to Associate Prof. Dr. Önder Albayram and Prof. Dr. Adviye Ergül for their valuable support. Special thanks are also due to MUSC for granting access to their research facilities.

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