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Research Article

mTOR Signaling Pathway Genes Effect in COVID-19 Infection

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ABSTRACT: Coronavirus disease 2019 (Covid-19) is an infectious disease that causes severe acute respiratory illness caused by coronavirus 2 (SARS-CoV-2). SARS-CoV-2 uses host-specific metabolic pathways, including mTOR. The mTOR pathway is hyperactive in viral respiratory tract infections and contributes positively to viral replication. 100 samples were evaluated, 50 patients (Female=23, Male=27), and 50 controls (Female=29, Male=21). The patients were individuals who were COVID-19 positive. We detected expression changes of 5 genes in mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (*MLST8*, *mTOR*, *RPTOR*, *MAPKAP1,* and *RICTOR*). Serum samples were obtained from all patients. The expression changes of mTORC1 and mTORC2 Complex genes were evaluated with the Real-time PCR method. Receiver operating curve (ROC) analysis was performed to define the diagnostic power of these genes. Expression changes of five genes in the mTORC1 and mTORC2 complex were statistically significant (p =0.001) and upregulated in serum. The area under the ROC Curve values indicating the diagnostic power of genes were 0.948, 0.771, 0.851, 0.798, and 0.805, respectively. The increased expression of these genes in the mTOR pathway used by SARS-CoV-2 in viral replication during the infection process shows that these genes and protein products are candidates for treatment targets. At the same time, the high discriminative power of these genes in patients from controls indicates their diagnostic potential in serum samples.

Keywords: *COVID-19, SARS-CoV-2, mTOR complex 1 (mTORC1), mTOR complex 2 (mTORC2)*

1 INTRODUCTION

Coronavirus is a virus from the Coronaviridae family [1]. The virus encodes four main structural proteins: Spike (S), Membrane (M), Envelope (E), and Nucleocapsid (N) [2]. The S protein is a type I transmembrane protein expressed on the virus surface and is responsible for the virus binding to its receptor in the target cell and entry into the cell [3]. S protein binds to receptors of coronavirus, and its ability to induce neutralizing antibodies in vivo has been reported [4]. The coronavirus infects mammals and birds, causing respiratory illness and symptoms such as diarrhea [5].

Severe acute respiratory syndrome (SARS) infection is potentially fatal in humans [6]. SARS infection is a disease that occurs with shortness of breath and high fever that

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turns into pneumonia [7]. The emergence of Covid-19 disease, caused by the new type of coronavirus called SARS-CoV-2, has posed a pandemic threat to public health. Covid-19 infection is a novel coronavirus causing 2,073,361 confirmed deaths worldwide by August 2022 (https://www.who.int/europe/emergencies/situations/covid-19). Therefore, researchers began to investigate the pathophysiology of the disease caused by the virus to uncover possible treatment methods for this disease and to identify therapeutic agents that may be effective against the disease.

Recent studies have focused on signaling pathways. One of these pathways, the mTOR signaling pathway, is involved in the basic cellular processes of transcription, protein synthesis, and cell metabolism [8]. The mTOR signaling pathway is regulated by interferons as part of the anti-viral response during viral infections [9]. In vitro, studies have shown that kinase inhibitors target the mTOR signaling pathway and reduce the spread of MERS-COV [10]. In addition, these studies suggest that the mTOR signaling pathway may be a potential drug target.

In this study, which we planned from this point of view, we quantitatively evaluated the changes in the expression of five genes in the mTOR signaling pathway in Covid-19 patients. We aimed to detect genes with changes in their activities and to evaluate their diagnostic power. Thus, it will contribute to the elucidation of the molecular mechanisms related to the pathophysiology of Covid-19 disease. In addition, the use of mTOR inhibitors in the treatment of Covid-19 disease will come into prominence. The use of a drug that developed against coronavirus, an RNA virus, for other RNA viruses may come into question.

2 MATERIAL AND METHOD

Population data and clinical epidemiology

The study group consisted of 50 patients and 50 healthy control individuals who applied to Malatya Turgut Ozal University, Faculty of Medicine, Department of Chest Diseases. The subjects have given their written informed consent.

Typically, patients have a fever, shortness of breath, cough, weakness, pain, dizziness, nausea, headache, vomiting, anorexia, chest pain, and back pain. The patients applied to the clinic for one, several, or more of these clinical histories. Patients were diagnosed with COVID-19 tests compatible with CT (Computed tomography) and diagnosed (they were experiencing the disease for the first time). Oxygen saturation was between 87-98. Oxygen therapy has been given by nasal, reservoir, intubated, nasal oxygen + chamber, reservoir + CPAP, and mask. Diseases that accompany the COVID-19 infection were seen as ischemic cerebrovascular disease (CVD) and

hypertension (HT), diabetes mellitus (DM) and immune tolerance, congenital adrenal hyperplasia (CAH), chronic renal failure (CRF), hemorrhagic CVD, ischemic CVD, asthma, pneumonia, ankylosing spondylitis, acute kidney failure (ARA), chronic obstructive pulmonary disease (COPD), subarachnoid hemorrhage (SH), schizophrenia, and asthma. Control subjects were PCR-negative with a clinical history similar to COVID-19-positive patients. The whole peripheral blood samples (5 ml) from patients and healthy controls were collected in gel tubes, centrifuged at +4 °C, and serum samples were stored at -80 °C.

Inclusion and exclusion criteria

Fever-decreased lymphocyte count, early-stage chest radiology and ground-glass opacities, bilateral inflammation, and epidemiological history were among the inclusion criteria. In addition, upper respiratory tract specimens, including nasopharyngeal, oropharyngeal, and nasal swabs were collected, and these materials were part of routine diagnostics to detect SARS-CoV-2 [11]. Individuals who did not have acute fever (>37.5°C) in the last 72 hours and had typical lung imaging were confirmed by repeated measurements and were excluded from the patient group. Clinical data and rapid deaths identified in the light of the data obtained during the pandemic period required urgent diagnosis and production of treatment protocols. For this reason, the boundaries of the disease were drawn based on the symptoms included in the inclusion criteria, since it is not a condition that has been encountered before and even if it is encountered, it does not result in sudden deaths [12]. With diagnostic tests entering the urgent phase, making a PCR diagnosis and starting treatment immediately became the best option. The difficulty in obtaining diagnostic kits forced clinicians to select patients with a preliminary diagnosis. As a result, patients who met the inclusion criteria were diagnosed with PCR and treated. In this context, individuals who applied to the hospital with these complaints had appropriate epidemiology and radiological findings were included in the study group.

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Why expression of mTOR complex genes

SARS-CoV-2 uses a host's metabolic pathway, such as mTOR, to synthesize virus particles. mTOR is an important treatment target because hyperactivation of the mTOR pathway contributes positively to viral replication. Investigation of the expression changes of mTORC1 and mTORC2 complex genes in our study will contribute to the evaluation of the infection process.

Determination of SARS-CoV-2

Many diagnostic kits were used to diagnose the disease during the pandemic process. Although there are still debates about their accuracy and effectiveness, kits that received FDA approval have been put into use. The important point in this study is that while these diagnostic tests are carried out rapidly in each region, it is important for this region as it allows the test results to be compared with others and is evidence for the success of the diagnosis. The diagnostic kit used for our patient group representing a region in Turkey is BIO-SPEEDY® DOUBLE GENE RT-QPCR KIT (BIO-SPEEDY, BS-SY-WCOR-307-250, bioeksen, Hindenburgstrasse, Germany). The feature of the kit is that it provides simultaneous detection of SARS-CoV-2 and Variants of Concern (VOC) (Alpha, Beta, Gamma, and Delta) in a single reaction. The test includes real-time, one-step reverse transcription PCR used for the qualitative detection of SARS-CoV-2-specific open reading frame 1 ab (Orf1ab), nucleocapsid (N), and human *ribonuclease P* (RNase P) gene fragments. There were occasional access problems for the modified kits, which are among the urgent solutions for pre-treatment diagnosis on a country basis, and they did not have the chance to be selected. Research on this kit, which is among the kits available and used in a regional hospital in Turkey, showed that this kit and other kits are similar in sensitivity and specificity [13].

RNA extraction and quantification

A total of 5 mL of *blood* was *drawn into* a serum *gel tube.* Total RNA was extracted from serum by RNeasy Mini Kit (Qiagen, Hilden, Germany) according to commercial protocol. RNA purity and quantification were performed with a spectrophotometer (MaestroNano Micro-Volume Spectrophotometer, MN-913, Maestrogen, Taiwan). The amount and purity of RNA from each sample were determined spectrophotometrically using a measuring range of 2~2000 ng/µL. 0.8 U of DNase I per 1 µg of RNA was used to purify RNA from DNA according to the kit protocol (Thermo Scientific™, EN0521, Waltham, MA USA) and incubated at 37°C for 30 minutes.

Complementary DNA (cDNA) synthesis

Complementary DNA was performed using an ABScript III RT Master Mix for qPCR Kit (ABclonal, United Kingdom). Reverse transcription (RT) reactions were prepared as 4µl 5xABScript III RT Mix, up to 1 ug total RNA, Nuclease-Free Water in a total of 20µl reaction. The Reactions were placed in an automated Gene Amp PCR System 9700 (Applied Biosystems, ABD) and incubated at 55°C for 15 minutes and at 85°C for 5 minutes. At the end of the period, they were immediately placed on ice and stored at -20 °C until further analysis.

Quantitative-Comparative CT (cycle thresold) (ΔΔCT) Real-time PCR (qPCR) analysis

Primers were synthesized (Synbio Technologies, USA) using SYBR Green probe technology. ACTB Human-Forward Sequence: 5′-CACCATTGGCAATGAGCGGTTC-3′ and ACTB Human-Reverse Sequence: 5′-AGGTCTTTGCGGATGTCCACGT-3′. MLST8 Human- Forward Sequence: 5′-CAGGTGAATGCCTTGGAGGTCA-3′ and MLST8 Human- Reverse Sequence: 5′-AGTCGATGCTGCCGCAGTTGTT-3′, mTOR Human-Forward Sequence: 5′AGCATCGGATGCTTAGGAGTGG-3′ and mTOR Human-Reverse Sequence: 5′-CAGCCAGTCATCTTTGGAGACC-3′, RPTOR Human-Forward Sequence: 5′-GATCGTCAACAGCTATCACACGG-3′ and RPTOR Human-Reverse Sequence: 5′-CGAGTCGAAGTTCTGCCAGATC-3′, RICTOR Human-Forward Sequence: 5′-GCCAAACAGCTCACGGTTGTAG-3′ and RICTOR Human-Reverse Sequence: 5′-GTCACCGAGTTACGAAGTAGACC-3′, MAPKAP1 Human-Forward Sequence: 5′-CAGGACAGACTGCTGCCAATGA-3′ and MAPKAP1 Human-Reverse Sequence: 5′-CTGTTACAGTCACGGATGACGG-3′. Real-time PCR mix was prepared as 2X SYBR Green reaction mix (ELK Biotechnology, China), 10 µM forward and

reverse primer, ddH2O, and 2 µl cDNA in total 10 µl reaction. RT-PCR methods were performed using a Real-Time PCR system RotorGeneQ (Qiagen, Hilden, Germany). Thermal cycling conditions were 30 seconds at 95°C followed by 40 cycles at 95°C for 5 seconds, 50-60°C for 30 seconds, and 72°C for 30 seconds. Melting curve stage added. All reactions were run in duplicate, and samples were normalized using the expression of the beta-actin (*ACTB*) housekeeping gene. Relative quantification of normalized samples was calculated according to the formula $2^{-\Delta\Delta Ct}$ and given as fold change value [14].

3 STATISTICAL ANALYSIS

According to the power analysis, the number of subjects in each group was calculated as 50 for a 0.20 difference between the two groups to be significant (Type I error = 0.05, power of the test = 0.8). Normality analyzes were performed with Kolmogorov-Smirnov and Shapiro-Wilk tests. ANOVA test was used for comparison between and within groups. Mann-Whitney U test was used to analyze the differences between groups. The Kruskal-Wallis test was used to measure the differences in gene expressions. Differences in mRNA were determined using the Bonferroni-corrected one-way ANOVA test for multiple comparisons at the $\alpha = 0.05$ cutoff point. The diagnostic values of the genes were evaluated by receiver operating characteristics (ROC) analysis. The diagnostic power of the genes was made according to Hosmer's rating [15]. In addition, logistic regression analysis was performed to determine the diagnostic values of these genes. Statistically, $p < 0.05$ was considered significant. Data were analyzed using the SPSS statistical package for Windows (Version 22.0, Armonk, NY: IBM Corp).

ROC analysis for mRNA diagnostic values

The ROC curve is a graphical representation of the relationship between sensitivity and selectivity. ROC analysis is an analysis method that contributes significantly to the clinical decision-making process. The area under the ROC curve determines the accuracy of the test in distinguishing patients from healthy controls, and the size of the area under the curve also indicates the statistical significance of the discrimination ability of the diagnostic test studied.

4 RESULTS

Clinical characteristics

The age and gender characteristics of the study population are given in Table 1. The number of males and females in the groups was approximately equal. Age and gender distribution in the groups are given in Figure 1. The mean age was 58.60 in the patient male, 66.13 in the patient female, 59.43 in the healthy male, and 55.28 in the healthy female. In the analysis of age distribution, 71 and 69 years were higher in the control and patient groups. In the multiple and comparative

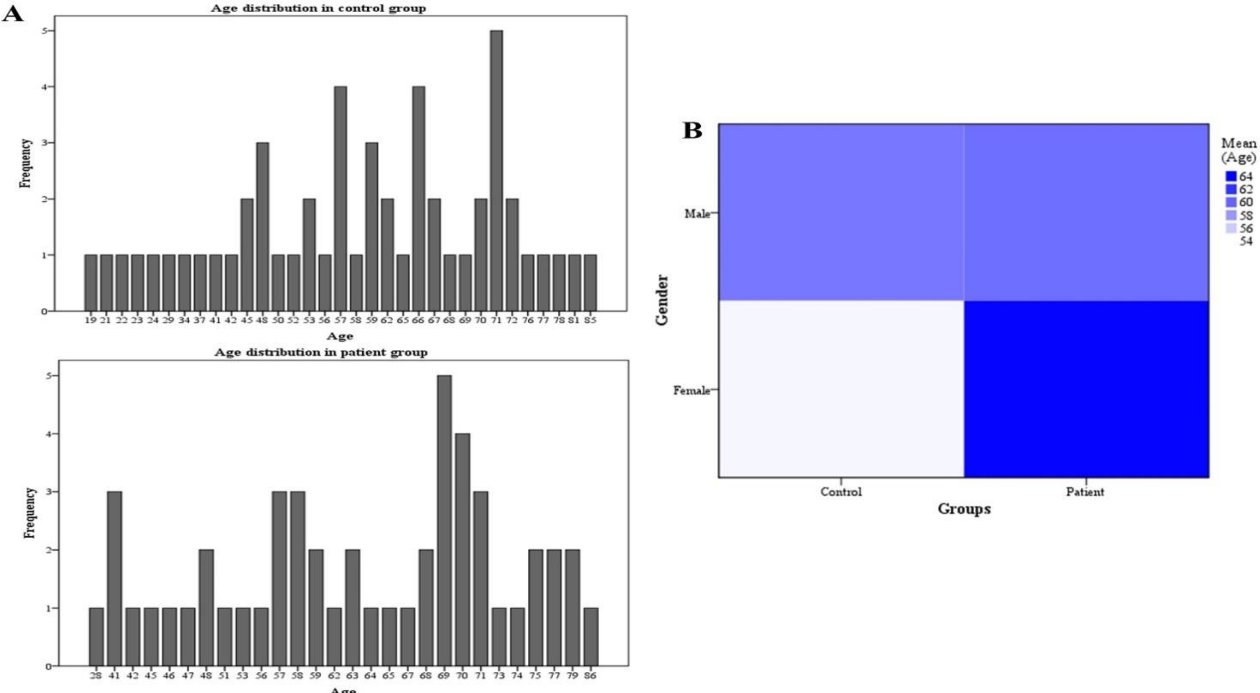

Figure 1. Analysis of age and gender factor in groups. In the age distribution analysis, it was observed that the 69-70 age group was high in the control and patient groups. Data are presented in groups as frequency and heat maps. **A.** Frequency representation of age distribution in the control and patient groups. **B.** Representation of age and gender distribution in the control and patient groups as means.

analyses performed, there was no statistical significance for age (p = 0.091) and gender (p = 0.234). Gene expressions were not associated with age and gender (p >0.05).

SARS-CoV-2 types our patient's groups.

Table 1. The characteristics of the two cohorts.

Covariates	Control (n)* (%)	Patient (n)** (%)
Age (years)	19-85	28-86
Gender		
Female	29 (58)	23 (46)
Male	21 (42)	27 (54)

*n = 50, **n = 50. NA; not applicable.

Pango lineage was expressed based on WHO records since they were samples according to the classification made at the beginning of the pandemic (https://www.who.int/publications/m/item/historical-working-definitions-and-primary-actions-for-sars- cov-2-variants). According to this (GSAID; Global Initiative on Sharing All Influenza Data);

Alpha ; Pango lineage-B.1.1.7, GISAID clade-GRY, nexstrain clade-20I (V1).

Beta; Pango lineage- B.1.351, GISAID clade- GH/501Y.V2, nexstrain clade- 20H (V2)

Gamma; Pango lineage- P.1, GISAID clade- GR/501Y.V3, nexstrain clade- 20J (V3)

Delta: Pango lineage- B.1.617.2, GISAID clade- G/478K.V1, nexstrain clade- 21A, 21I, 21J.

In the Omicron variant and subsequent classifications, Orf1ab and N positive mutations were determined as stated below (According to kit diagnostic targets).

BF.7; N: G30-, S33F

BQ.1; ORF1a: Q556K, L3829F, ORF1b: Y264H, N: E136D

BA.2.75 and CH.1.1; ORF1a: S1221L, P1640S, N4060S, ORF1b: G662S

XBB; ORF1a: K47R, ORF1b: G662S, S959P

Gene expression analysis

The expression analyses of the five genes belonging to the mTORC1 and mTORC2 complexes in COVID-19 patients and healthy individuals were found to be statistically significant ($p < 0.05$) (Table 2). In the descriptive statistical analyses performed to reveal the difference between COVID-19 patients and healthy control individuals, upregulation of five genes was detected in the patient group (Figure 2). We evaluated the contribution of these genes to Covid-19 disease by logistic regression analysis. All genes were modeled and analyzed. *mTOR* (OR = 1.088, 95% CI = 0.802-1.475, p = 0.001), *RPTOR* (OR = 1.154, 95% CI = 0.614-2.168, p= 0.001), *MAPKAP1* (OR = 1.160, 95% CI = 0.900-1.494, p=0 .001), *RICTOR* (OR = 0.991, 95% CI = 0.574-1.709, p = 0.001), and *MLTS8* was significant (OR = 2.288, 95% CI = 1.531-3.417, p =0 .0001).

ROC analysis for diagnostic values of five genes

The diagnostic value of all genes was graded high and statistically significant (Table 3) (Figure 3). According to the classification showing the diagnostic value of AUC values,[16] *MLST8* was found as "excellent" *RPTOR*, *RICTOR* "very good", *mTOR*, and *MAPKAP1* "good" (Table 4).

Table 2. Gene expression analysis five gene in case-control cohort (ANOVA).

Genes	Groups	N	Mean±SD	SE	95% CI		*P-value*
MLST8	Control	50	-4.446±2.164	0.306	-5.061	-3.830	**<0.001***
	Patient	50	1.416±2.837	0.401	0.610	2.222	
mTOR	Control	50	0.474±2.476	0.350	-0.230	1.177	**<0.001***
	Patient	50	3.413±3.239	0.458	2.492	4.333	
RPTOR	Control	50	-16.117±1.625	0.230	-16.579	-15.655	**<0.001***
	Patient	50	-13.359±2.141	0.303	-13.968	-12.750	
MAPKAP1	Control	50	0.322±3.046	0.431	-0.544	1.187	**<0.001***
	Patient	50	4.276±3.834	0.542	3.186	5.365	
RICTOR	Control	50	-1.578±1.837	0.260	-2.100	-1.055	**<0.001***
	Patient	50	0.970±2.403	0.340	0.287	1.653	

*P < 0.05 is significant. SD; standart deviation, SE; standart error, CI; confidence interval.

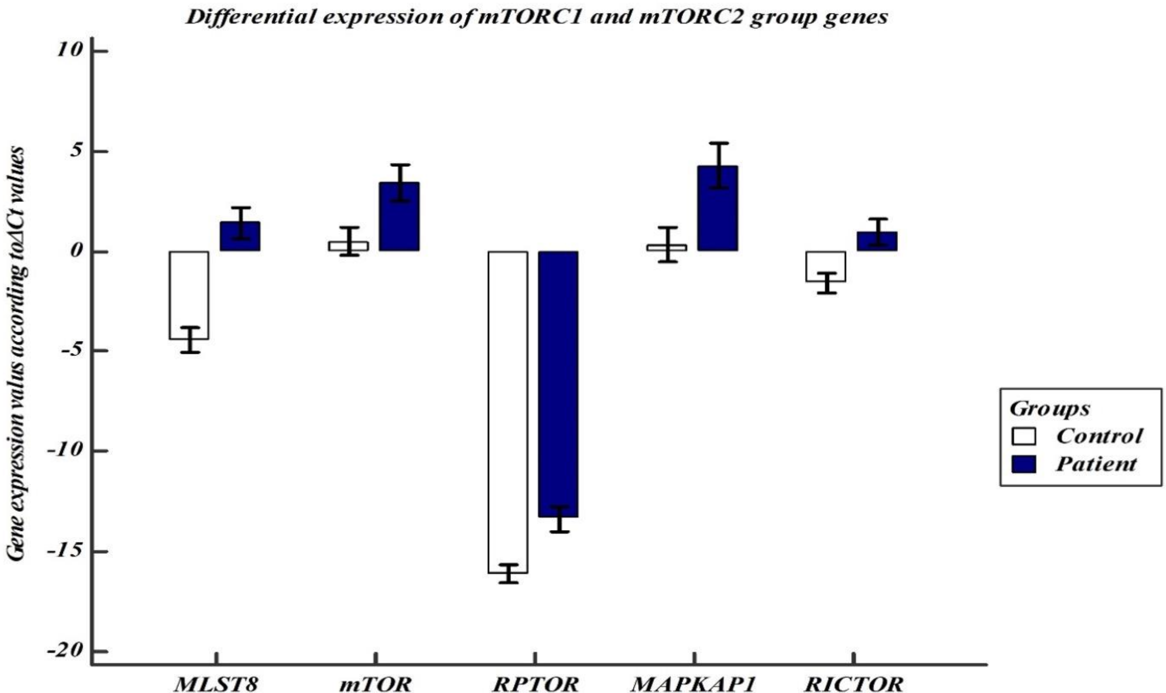

Figure 2. Differential gene expression analysis of five gene. The expression difference between COVID-19 patients and control subjects is presented in the graph. Expression values indicate the ΔCt. A Mann-Whitney U test was used to reveal the differences in miRNA expressions between groups. As a result of the analysis, a statistically significant difference was found between expressions and groups for five genes (P < 0.001).

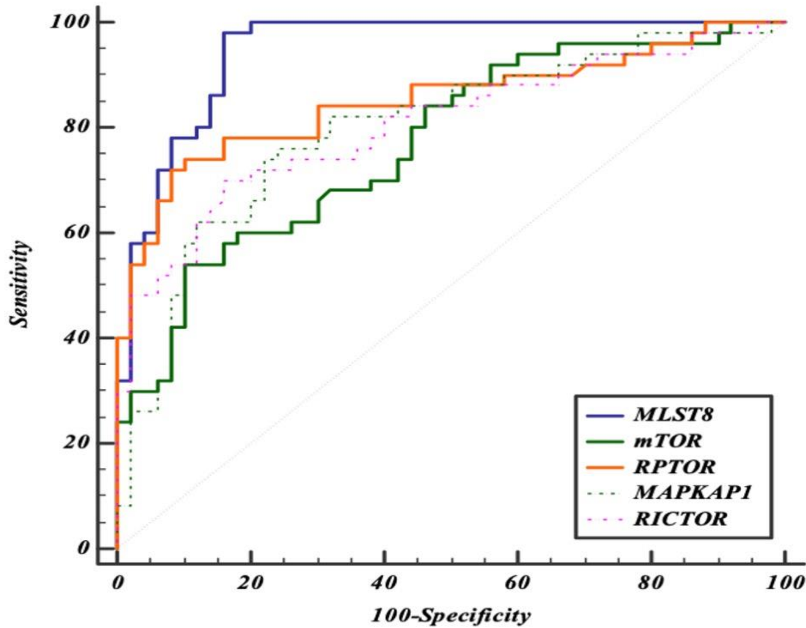

Figure 3. Roc curve analysis for diagnostic values of five genes. All AUC values were statistically significant (p<0.001).

Table 3. ROC curve analysis of five genes.

Compared Groups	Genes	AUC	95% CI	SE	P-Value	Specificity	Sensitivity	Criterion
Patient vs Control	*MLST8*	0.948	0.907-0.989	0.021	< 0.001	0.98	0.84	>-2.460
	mTOR	0.771	0.680-0.861	0.046	< 0.001	0.90	0.54	>3.255
	RPTOR	0.851	0.772-0.929	0.040	< 0.001	0.90	0.74	>-14.425
	MAPKAP1	0.798	0.710-0.886	0.045	< 0.001	0.76	0.76	>1.915
	RICTOR	0.805	0.718-0.892	0.044	< 0.001	0.84	0.70	>0.05

Table 4. Pairwase comparison of ROC curves.

Gene combinations	DBA	SE	95% CI	Z statistics	*P*-Value
MLST8 ~ mTOR	0.177	0.045	0.090-0.265	3.974	**0.000***
MLST8 ~ RPTOR	0.097	0.042	0.014-0.180	2.300	**0.022***
MLST8 ~ MAPKAP1	0.150	0.047	0.058-0.242	3.186	**0.001***
MLST8 ~ RICTOR	0.143	0.046	0.052-0.233	3.109	**0.001***
mTOR ~ RPTOR	0.080	0.049	-0.008-0.168	1.786	0.074
mTOR ~ MAPKAP1	0.027	0.066	-0.102-0.157	0.415	0.678
mTOR ~ RICTOR	0.034	0.056	-0.075-0.144	0.615	0.539
RPTOR ~ MAPKAP1	0.052	0.052	-0.049-0.154	1.019	0.308
RPTOR ~ RICTOR	0.045	0.031	-0.014-0.105	1.496	0.135
MAPKAP1 ~ RICTOR	0.007	0.046	-0.082-0.097	0.153	0.878

* $P < 0.05$ is statistically significant. SE; standard error, CI; confidence interval, DBA; the difference between areas.

5 DISCUSSION

COVID-19 has captured the world for a long time and has become a permanent disease due to its severe clinical symptoms and rapid spread. We don't know exactly how our immune system responds to Covid-19. Personal differences and underlying illnesses show that the process is individual. The vaccine and vaccination process, which cured the long-lasting COVID-19 disease in a short time, caused relief. In this process, much data was obtained for both diagnosis and treatment. However, several factors need to be identified to manage the disease. At this point, one of the most appropriate approaches would be studies to determine the markers and targets that can determine the genetic basis for this disease, whose course varies according to individual differences.

The most important reaction of COVID-19 is the severe inflammatory response seen in patients after infection and the cytokine storm in end-stage cases. Cytokine storm results in acute respiratory distress syndrome, multiple organ failure accompanied by lung and other organs, leading to death with progressive COVID-19. Activation of inflammatory pathways is the main factor here

[17]. Viral entry of the spike protein on the surface of the virus involves binding to host cell receptors and viral replication. As a result of the activation of the mTOR pathway by the Warburg effect, COVID-19 replication and inflammatory response are promoted. Thus, viral protein synthesis increases and cytokine production is forced. Therefore, mTOR is an important therapeutic target in COVID-19.

The virus uses a host metabolic mTOR pathway for particle synthesis. The reason why the virus targets a powerful pathway such as mTOR is that this signaling pathway is involved in mRNA translation [18]. The source of this power of the mTOR pathway is its ability to control metabolic events such as autophagy, cell proliferation, protein synthesis, cellular growth, proliferation, insulin, and response to oxygen [10]. This situation shows us the importance of the mTOR pathway in respiratory failure observed in COVID-19 patients and the presence of covariant diseases such as hypertension, cardiovascular disease, and diabetes. Clinical studies also show hyperactivation of the mTOR pathway during infection [19]. Investigating genes in the mTOR pathway, which has a significant role in COVID-19, may be a target in diagnosis and treatment. Rapamycin is the best-known mTORC1 inhibitor [20]. More potential targets need exploring to find durable solutions. mTOR has two protein complexes, mTORC1 and mTORC2 [8]. In both complexes, mTOR is the central protein. The mTORC1 complex includes mTOR, DEPTOR, PRAS40, RPTOR, FKBP12 rapa, and MLST8. RPTOR targets HEAT (Huntingtin, elongation factor 3, the A subunit of protein phosphatase 2A (PP2A), and the signaling kinase TOR1)[21] (usually involved in intracellular transport systems)[22] repeats while MLST8 targets the kinase active site. The mTORC2 complex includes DEP domain-containing mTOR-interacting protein (DEPTOR), Protor1/2, mSin1 (MAPKAP1), RICTOR, and MLST8. RICTOR targets HEAT repeats, while MLST8 targets the kinase active site.

Sirolimus, everolimus, and metformin are the detected inhibitors of the mTOR complex, and with this inhibition, cell cycle progression, transcription, translation, and protein synthesis are targeted [23]. mTOR complex of genes may be a new target for diagnosis and treatment in patients with Covid-19. Gene expression studies for COVID-19 refer to dysregulated regulation of various genes. Moni et al. compared SARS-CoV-2 with SARS-CoV, MERS-CoV, and influenza A strains H1N1, H3N2, and H5N1 in their genomic and transcriptomic analyses. They detected a striking upregulation for 40 SARS-CoV-2 infection response genes in patients' peripheral blood samples compared to other viruses [24]. Comorbidities with a disease can distort results and hinder the purification of the

target. Excluding comorbidities, multi-omic analyses using artificial intelligence in young patients detected up-regulation of metalloprotease and ADAM9 among genes with different expressive expressions [25]. The planned ex vivo study showed that inhibition of ADAM9 reduces SARS-CoV-2 uptake and replication in human lung epithelial cells. In a study investigating the reason for the low risk of Kovid-19 in children compared to adults, it was stated that the angiotensin-converting enzyme 2 (ACE2) receptor, which SARS-CoV-2 uses when entering the host, is expressed differently [26]. In a cohort study of 305 individuals aged 4 to 60 years, 49.8% of individuals had asthma. The result was that ACE2 expression was lowest in young children and higher with increasing age. It can be said that the effects of Covid-19 symptoms in the middle-aged group increase depending on certain genes. With the contribution of comorbid factors in advanced ages, the patient's condition gets worse and may result in death. Differential gene expression analyses of COVID-19 severity showed differential expression of 55 genes according to disease severity [27].

The *Orf 1ab*, *N*, and *Rnase P* gene regions are important targets in the viral process. The relationship between positive mutations in these targets and mTOR in patients was demonstrated for the first time in this study. Previous data report the association of mTOR and autophagy pathways with disease severity [28]. Additionally, increased mTOR signaling and comorbidities that pose a higher risk of mortality due to Sars-CoV-2 are common [29]. The mTOR pathway was suggested among the drug targets identified in interactome studies for Sars-CoV-2 [30]. The relationship of mTOR with autophagy is known. Disruption of autophagic circulation in cells infected with SARS-CoV-2 causes increased virus replication [31]. These results may be associated with serious symptoms of COVID-19.

Studies indicate that the expression of genes in the mTOR complex has a high effect potential in COVID-19 patients. Indeed, as we demonstrated in our study, *MLST8*, *mTOR*, *RPTOR*, *MAPKAP1*, and *RICTOR* genes were upregulated in our patient group with different comorbidities. Consistent with other studies, the hyperactivity of the mTOR pathway in COVID-19 is due to overexpression of the responsible proteins. mTOR pathway activation contributes to viral replication. It is currently a holistic solution to combating disease, as treatments are based on inhibiting the mTOR complex or PI3K or AKT. Targeting genes in the complex may be a more specific and effective approach. Epigenetics or phytotherapeutics can also offer effective solutions for this. It can also reduce the extra burden of drug side effects and comorbidities. The contribution of gene expressions to the

disease process is the first pillar of the research, and the second pillar is the diagnostic potential. Diagnostic analyses demonstrated that *MLST8* has the potential as a marker to distinguish patients from controls. *RPTOR* and *RICTOR*, are in second place, followed by *MAPKAP* and *mTOR* detected as significant markers. Age, gender, and comorbidities are not significant, but since the age range studied is young and old populations. In studies conducted for age and gender in COVID-19, it was seen that the mortality rate was higher in male patients and the risk was higher due to the added comorbidities with advancing age (i.e., diabetes, hypertension, cardiovascular disease, etc.) [32, 33].

6 CONCLUSIONS

In this study, we present information on the activity of five genes in the mTOR pathway in the cellular response to SARS-CoV-2. Additional biochemical and clinical studies are needed to demonstrate the role of mTOR inhibitors and modulators in treating COVID-19.

7 FUNDING

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8 CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

9 ETHICAL APPROVAL

This study has been approved by the clinical research ethics committee of Malatya Turgut Ozal University (2021/27).

10 AUTHORS CONTRIBUTIONS

M.P. and S.G.Y. designed the study; M.P and E.S.T conducted experimental studies; A.G., L.A.D, D.A.O and M.O. collected patient data; M.P. and S.G.Y. prepared the initial manuscript; M.P. and S.G.Y. edited the initial version of the manuscript; all authors approved the final version of the manuscript.

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Case Report

Lithium Toxicity Due to Drug-Drug Interactions: A Case Report

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ABSTRACT: Lithium has a relatively narrow therapeutic range. As a result of drug-drug interactions, the blood level of lithium is highly affected. Drug-induced lithium toxicity and therapy are discussed in this case. A 37-year-old woman with bipolar illness, essential hypertension, hypothyroidism, unipolar depression, and schizoaffective disorder was admitted to the emergency department with hand numbness, tremor, agitation, and tachycardia. Due to a history of lithium use, her blood lithium concentration was measured and found to be 1.7 mmol/L. As a result, lithium treatment was suspended, and she was hospitalized in the intensive care unit with a diagnosis of lithium toxicity. Lexicomp® drug interaction database was examined by the clinical pharmacist. Candesartan, hydrochlorothiazide, and dexketoprofen caused toxicity. The clinical pharmacist advised stopping the existing antihypertensive regimen and starting amlodipine, as it did not interact with lithium, reducing its levels to 1.4-1.2 and 0.8 mmol/L, respectively. Clinical pharmacist treated drug-induced lithium toxicity.

Keywords: *drug-drug interactions, lithium, pharmacy, toxicity*

1 INTRODUCTION

Lithium is a mood-stabilizing medication used primarily in the treatment of bipolar disorder (1). In the treatment of bipolar disorder, the therapeutic drug trough level of lithium is desired to be between 0.8-1.2 mmol/L in acute mania episodes and 0.6-1 mmol/L in maintenance treatment (2). In this case report, a patient who developed lithium toxicity due to drugs interacting with lithium and the management of drug-drug interactions is presented. Informed consent was obtained from the patient for this study.

2 CASE PRESENTATION

A 37-year-old woman with a history of bipolar affective disorder (8 years), essential hypertension (5 years), hypothyroidism (4 years), unipolar depression (9 years) and schizoaffective disorder (1 year) presented with numbness and tremor in her hands, agitation and tachycardia. The patient was referred to the emergency room by her doctor due to her condition. Based on the patient's and her relatives' anamnesis, it was determined that the patient had no further complaints and maintained a regular sleep pattern. When

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the mental status examination was examined, it was seen that she was conscious, oriented and cooperative. Her associations were regular; her speech rate and amount were normal; she had no suicidal or homicidal thoughts; and she did not describe any delusions. The lithium blood level was found to be 1.7 mmol/L. Following the diagnosis of lithium toxicity, the patient was admitted to the anaesthesiology and reanimation intensive care unit (ICU) and lithium treatment was suspended. When the patient was admitted to the intensive care unit, her vital signs were as follows: blood pressure: 128/68 mmHg, pulse rate: 121 beats/min.

The medications used routinely by the patient were given together with their indications in the ICU:

- Aripiprazole 10 mg every 24 hours (q24h) and lithium carbonate 300 mg every 8 hours (q8h): Bipolar affective disorder
- Candesartan cilexetil + hydrochlorothiazide 32 mg/12.5 mg q24h: Essential hypertension
- Levothyroxine sodium 100 mcg q24h: Hypothyroidism
- Quetiapine fumarate 50 mg q24h: Schizoaffective disorders
- Dexketoprofen 25 mg: She takes it on demand every time she has pain

The potential drug-drug interactions were reviewed by clinical pharmacists using the Lexicomp® Drug Interaction Checker. Three drug-drug interactions with a risk level of D that could increase lithium blood concentrations were identified. These:

1. Lithium + Candesartan: Although the interaction mechanism is not fully known, angiotensin receptor blockers increase the retention of lithium by inducing natriuresis. In patients taking angiotensin II receptor blockers (ARBs), it is recommended to start lithium at lower doses.
2. Lithium + Hydrochlorothiazide: Although the mechanism of interaction is not fully known, hydrochlorothiazide increases the proximate tubular reabsorption of lithium.
3. Lithium + Dexketoprofen: Although the mechanism of interaction is not fully known, NSAIDs (nonsteroidal anti-inflammatory drugs) reduce the renal clearance of lithium.

The clinical pharmacist reported these drug-drug interactions to the physician in charge of the ward and recommended that the current antihypertensive medication be discontinued for the duration of the ward stay and amlodipine 10 mg q24h be given as an antihypertensive that does not cause any interaction, which the physician did. Samples were taken for lithium blood levels the day after the change in treatment. These showed concentrations of 1.4–1.2 and 0.8 mmol/L, respectively. Lithium produces a therapeutic response with serum concentrations between

0.8 and 1.2 mmol/L in a patient receiving 300 mg three times daily, as in this case. It was unusual for this patient to have an initial measured lithium serum concentration of 1.7 mmol/L. Discontinuation of lithium treatment showed that lithium concentrations had decreased and remained at therapeutic levels. After discharge, the patient's lithium treatment was continued by the physician in charge.

In a written consultation note given to the patient and her relative by the clinical pharmacist, the following recommendations were made.

It was said that the increase in lithium levels occurred due to drug interactions and that the drugs causing these were candesartan + hydrochlorothiazide, an antihypertensive that the patient used routinely and dexketoprofen, which she used whenever she had pain.

Alternatively, as the patient's previous use of amlodipine caused ankle edema, the patient was told that lercanidipine 10 mg or 20 mg could be given if necessary as an antihypertensive that does not interact with lithium and causes less edema (3), and that she could use acetaminophen rather than dexketoprofen when she had pain. The patient was discharged on the third day of hospitalisation with improvement in symptoms. When the patient was contacted 1 week after the discharge, she shared the consultation note written by the clinical pharmacist with her doctor during the internal medicine clinic control and it was learnt that lercanidipine 10 mg q24h was prescribed as antihypertensive.

3 DISCUSSION

Currently, lithium is considered the primary choice for preventing bipolar disorder. However, using this medicine safely and effectively requires a thorough understanding of its pharmacokinetics, potential adverse effects, and the relative risk of drug-drug interactions (4). Lithium blood levels may vary depending on drug-drug interactions. While some interactions reduce lithium blood levels, some interactions lead to an increase, and as a result of this increase, intoxication may even occur. Our patient also had tachycardia and numbness in the hands due to lithium toxicity that developed as a result of drug-drug interactions. Drug groups that cause an increase in lithium blood levels include diuretics, NSAIDs and angiotensin-converting enzyme (ACEi) inhibitors (5). Lithium levels showed a clear relationship with gender, that women having higher values. However, only NSAIDs were found to have the ability to independently increase lithium levels (6). Although the interactions between these drugs and lithium are not contraindicated, lithium levels should be closely monitored due to the risk of occurrence of changes in lithium blood levels.

Due to the narrow therapeutic range, even slight changes in lithium concentration can cause serious side effects. Many case reports in the literature emphasize that ACEi (7), NSAIDs (8) and thiazide diuretics (9) may increase lithium reabsorption and thus increase blood lithium concentrations. In this case, several medications may have played a role in lithium toxicity. The patient was on a combination of candesartan and hydrochlorothiazide. Since the medications in this combination have a similar effect in increasing lithium blood concentrations, they exacerbate the situation. In addition, the patient was taking NSAID pain relievers. NSAIDs decrease glomerular filtration rate by inhibiting prostaglandin synthesis. In our case, these mechanisms led to decreased lithium excretion and increased blood levels. This toxicity was effectively managed by changing the drugs.

4 CONCLUSION

Based on the presented case, it is understood that patients receiving lithium therapy should be informed about drug-drug interactions and that caution is required. Due to the narrow therapeutic dose range of lithium, it is important to closely monitor it for toxicity and carefully evaluate any reported indications of toxicity. Attention should be paid to the initiation of additional medication, or the use of non-prescribed medication and it should be remembered that the patient should be informed about the symptoms of poisoning. Pharmacists play a crucial role in minimizing the potential negative consequences of drug-drug interactions through providing of information to patients, their relatives, and healthcare professionals.

5 AUTHOR CONTRIBUTIONS

Design: M.S.T., K.K.; Resources M.S.T., A.Ç.; Data collection: M.S.T.; Writing: M.S.T., K.K., A.Ç., N.Ö.A.; Critical Review: A.Ç., N.Ö.A.

6 CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Research Article

Antimicrobial Resistance of E. coli Strains Isolated from Urine Cultures

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ABSTRACT: Urinary tract infections (UTIs) are among the most common infectious diseases. This study retrospectively analyzed the antimicrobial resistance rates of E. coli strains isolated from urine culture samples sent to the Pamukkale University Health Research and Application Hospital between July 12, 2023, and July 12, 2024. A total of 1,844 urine culture samples were analyzed. Of the isolates, 68.54% were from female patients, and 31.46% were from male patients. The highest resistance in antibiotic susceptibility tests was observed against drugs such as ampicillin (67.22%), cefazolin (51.62%), and cefuroxime (48.09%). The lowest resistance rates were found against tigecycline (1.12%), ceftazidime-avibactam (1.28%), imipenem, and meropenem (2.28%). Particularly, higher resistance rates to antibiotics were noted in intensive care units, which could be related to the more frequent use of antibiotics in these units. Antibiotics such as tigecycline and ceftazidime-avibactam exhibited relatively low resistance rates in intensive care.

Keywords: *antibiotic resistance, E. coli, urinary tract infections*

1 INTRODUCTION

Urinary tract infections (UTIs) are the most frequently occurring infectious diseases [1]. It is estimated that 10-12% of women experience at least one UTI annually [2]. Escherichia coli is the most common causative agent in the etiology of UTIs. Antibiotics are often initiated empirically in the treatment of UTIs. Trimethoprim-sulfamethoxazole, cipro-floxacin, and beta-lactam antibiotics are frequently used antibiotics in treatment [3]. There is an increasing trend of resistance to antibiotics that are initiated empirically [4].

The development of antimicrobial resistance in bacteria poses a significant problem in antibiotic selection and reduces treatment success rates [5].

The antibiotic resistance profiles of bacteria isolated in UTI cases can vary due to the rapid development of resistance. Therefore, it is essential to keep empirical treatment options updated in UTI cases [6].

Our study aims to evaluate the antimicrobial resistance of E. coli strains

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isolated from urine cultures.

2 MATERIAL AND METHOD

The antimicrobial resistance rates of *E. coli* strains isolated from urine culture samples sent to the Pamukkale University Health Research and Application Hospital Medical Microbiology Laboratory between July 12, 2023, and July 12, 2024, were retrospectively analyzed. Only the first isolate from multiple samples belonging to the same patient was included in the evaluation. Repeated samples were excluded from the study.

2.1 Identification of Bacteria and Antibiotic Susceptibility Testing

The urine culture samples sent to our laboratory were inoculated onto 5% sheep blood agar and "Eosine Methylene Blue" (EMB) agar (Becton Dickinson, USA). They were incubated at 37°C for 18-24 hours in an incubator. The growths in the cultures were evaluated according to guideline recommendations. Samples with uropathogenic growth of $\geq 10^3$ cfu/ml in invasive samples and $\geq 10^4$ cfu/ml in non-invasive samples ($\geq 10^3$ cfu/ml in women of reproductive age) were included in the evaluation. For the identification of bacteria growing in culture samples, traditional methods or the Bruker MALDI Biotyper (Bruker Daltonics, Bremen, Germany) automated identification system was used.

The Kirby Bauer disk diffusion method and the Phoenix™ (Becton Dickinson Diagnostics, USA) automated system were used to determine the antibiotic susceptibility of isolates identified as *E. coli*. The antimicrobial susceptibility of isolates to ampicillin, amoxicillin/clavulanic acid, piperacillin/tazobactam, cefazolin, cefotaxime, cefepime, ceftazidime, cefuroxime, ertapenem, imipenem, meropenem, gentamicin, amikacin, tobramycin, ciprofloxacin, trimethoprim/sulfamethoxazole, ceftazidime-avibactam, tigecycline, colistin, and fosfomycin was tested using the automated system and Kirby Bauer disk diffusion method. Antibiotic susceptibility results were evaluated according to "The European Committee on Antimicrobial Susceptibility Testing" (EUCAST) criteria [7].

This study was conducted with the approval of the Pamukkale University Non-Invasive Research Ethics Committee (Date: 06.08.2024 and Number: E-60116787-020-563968).

3 RESULT

A total of 1,844 urine culture samples sent to the Medical Microbiology Laboratory of the Health Research and Application Hospital between July 12,

2023, and July 12, 2024, were considered significant and subjected to bacterial identification and antimicrobial susceptibility testing. The age range of the patients was min. 0-max. 94 years. Of the *E. coli* isolates, 1264 (68.54%) were from female patient samples, and 580 (31.46%) were from male patient samples. Of the 1844 *E. coli* isolates included in the study, 1358 (73.64%) were from outpatient clinics, 430 (23.31%) from wards, and 56 (3.03%) from the intensive care unit. The antimicrobial agent with the highest resistance rate was ampicillin (67.22%), followed by cefazolin (51.62%), cefuroxime (48.09%), and cefotaxime (43.47%). The lowest resistance rates were observed against tigecycline (1.12%), ceftazidime-avibactam (1.28%), imipenem, and meropenem (2.28%).

The resistance rates for the carbapenems meropenem and imipenem were found to be the same (2.28%), while the resistance rate for ertapenem was higher (5.6%). The antimicrobial resistance percentages of the isolates are presented in Table 1.

Table 1. The antimicrobial resistance rates of *Escherichia coli* isolates (74 outpatient; 23 ward; 3 intensive care) isolated from 1,844 patients included in the study are [% (n)].

Antibiotic	Total (n=1844)	Outpatient Clinic (n=1358)	Inpatient (n=430)	Intensive Care Unit (n=56)
Amoxicillin/Clavulanic Acid	43.26 (764/1766)	40.32 (523/1297)	51.93 (215/414)	47.27 (26/55)
Ampicillin	67.22 (1124/1672)	62.84 (783/1246)	80.58 (303/376)	76 (38/50)
Ceftazidime-Avibactam	1.28 (15/1164)	0.69 (6/865)	2.29 (6/262)	8.10 (3/37)
Tigecycline	1.12(16/1418)	0.75 (8/1056)	1.88 (6/319)	4.65 (2/43)
Fosfomycin	9.18 (97/1056)	9.29 (74/796)	8.69 (20/230)	0.33 (3/30)
Ciprofloxacin	33.78 (595/1761)	28.33 (367/1295)	48.54 (200/412)	51.85 (28/54)
Imipenem	2.28 (42/1837)	1.70 (23/1352)	3.49 (15/429)	7.14 (4/56)
Ertapenem	5.6 (60/1070)	3.99 (32/801)	9.66 (23/238)	16.12 (5/31)

Meropenem	2.28 (42/1839)	1.47 (20/1356)	4.21 (18/427)	7.14 (4/56)
Gentamicin	17.23 (311/1804)	14.50 (193/1331)	23.44 (98/418)	36.36 (20/55)
Amikacin	3.15 (58/1839)	2.65 (36/1355)	4.43 (19/428)	5.35 (3/56)
Piperacillin/Tazobactam	14.36 (204/1420)	12.39 (130/1049)	19.03 (63/331)	27.5 (11/46)
Trimethoprim/Sulfamethoxazole	38.84 (677/1743)	36.22 (468/1292)	48.36 (192/397)	31.48 (17/54)
Cefazolin	51.62 (207/401)	46.55 (135/290)	62.88 (61/97)	78.57 (11/14)
Cefuroxime	48.09 (797/1657)	42.93 (532/1218)	62.21 (242/389)	64 (32/50)
Cefotaxime	43.47 (736/1693)	38.70 (483/1248)	56.85 (224/394)	56.86 (29/51)
Ceftazidime	42.44 (683/1609)	37.71 (445/1180)	55.52 (211/380)	55.10 (27/49)
Cefepime	28.48 (476/1671)	24.09 (300/1245)	41.37 (156/377)	40.81(20/49)

4 DISCUSSION

E. coli is among the most commonly isolated uropathogenic agents in urinary tract infections (UTIs), and fluoroquinolones, trimethoprim-sulfamethoxazole (TMP-SMX), or beta-lactam antibiotics are widely used as the first-line empirical treatment for UTIs. In recent years, a significant increase in resistance rates to commonly used antibiotics such as fluoroquinolones and TMP-SMX has been reported. The Infectious Diseases Society of America (IDSA) recommends that if the resistance rate to TMP-SMX in a region exceeds 20%, this agent should not be used as the empirical treatment for urinary infections [8]. Studies conducted in our country have reported resistance rates of 21% to 60% to TMP-SMX and 7% to 41% to fluoroquinolones in E. coli strains isolated from urine [9]. In our study, resistance rates of 39% to TMP-SMX and 34% to ciprofloxacin were observed, which are consistent with some studies conducted in our country. Considering the literature recommendations, the results of studies conducted in our country, and our findings, we conclude that the use of fluoroqinolones and TMP-SMX as empirical treatment for

UTIs may not be appropriate.

According to the IDSA guidelines, nitrofurantoin and fosfomycin are recommended for the treatment of uncomplicated urinary tract infections due to their oral convenience and low resistance rates [8]. In our country, data on fosfomycin resistance rates in E. coli strains have been analyzed in a limited number of studies. The analysis by Pullukçu et al. [10] reported an average fosfomycin resistance rate of 1.9% for a total of 6,439 isolates [10]. In a study conducted by Gündüz et al. [11], fosfomycin resistance was found to be 3.8% in 10,709 isolates. In our study, a resistance rate of 9.18% was observed. Resistance rates were 9.29% in outpatient clinics, 8.69% in wards, and considerably lower at 0.33% in the intensive care unit. The low fosfomycin resistance in the intensive care unit may be related to its less frequent use in these settings. Compared to other studies, our fosfomycin resistance rate is higher. However, since the resistance rate is below 20%, fosfomycin can still be considered a viable option for empirical treatment.

Carbapenems are frequently preferred antibiotics for the treatment of broad-spectrum beta-lactamase positive bacteria. However, inappropriate use of these agents has become a significant issue leading t o the development of resistance to carbapenems. An evaluation of our study and other literature reveals that carbapenem resistance rates, especially in intensive care units, have reached concerning levels. In a 2022 study by Aygar et al. [12], imipenem resistance was reported to be 7.3%. In our study, imipenem resistance was found to be 1.7% in outpatient clinic patients, 3.49% in ward patients, and 7.14% in intensive care unit patients. We believe that the higher resistance rate in intensive care unit patients may be associated with a greater use of imipenem for treatment in these units compared to other departments.

In our study, the resistance rate to tigecycline was found to be 1.12% overall, making it the antibiotic with the lowest resistance rate. We observed resistance rates of 0.75% in outpatient isolates, 1.88% in ward isolates, and 4.65% in intensive care unit isolates. We believe that the higher resistance rate in intensive care units compared to other departments may be attributed to the lack of an oral formulation of tigecycline, which prevents its frequent prescription for outpatient treatment. In a study by Alanli et al., the tigecycline resistance rate was determined to be 2% [13]. This finding is consistent with our results and indicates that there is still no high resistance rate to tigecycline.

Ceftazidime-avibactam is a combination of ceftazidime, a third-generation broad-spectrum cephalosporin, and avibactam, a β-lactamase inhibitor. Avibactam is a β-lactamase inhibitor with a diazabicyclooctane structure that does not have a β-lactam structure. This combination is effective against a broad spectrum of Gram-negative bacteria, including carbapenem-resistant Enterobacteriaceae and Pseudomonas aeruginosa [14]. In a study by Bilgin et al. [15], the resistance rate to ceftazidime-avibactam in E. coli isolates was found to be 5%. In our study, the overall resistance rate was 1.28%, with 0.69% in outpatient clinics and 2.29% in wards. However, in intensive care units, this rate reached a high value of 8.10%. We believe that the higher resistance rate in intensive care units may be related to the more frequent use of ceftazidime-avibactam in these units compared to other departments.

Since penicillins are not resistant to beta lactamases, their susceptibility has been increased by combining them with beta lactamase inhibitors. Yüksel G et al. reported piperacillin/tazobactam susceptibility as 90.95% in outpatients and 86.95% in ward patients [16]. It was compatible with the data of our study.

In intensive care units, resistance rates for most antibiotics are noticeably higher compared to other clinical settings. This may be a result of more frequent and intensive empirical use of antibiotics in intensive care patients or due to more severe infections. Antibiotics such as amoxicillin/clavulanic acid, ampicillin, fosfomycin, and trimethoprim/sulfamethoxazole exhibit low resistance rates in intensive care units. This situation may be due to the less frequent use of these oral medications in intensive care settings.

Conclusion: The findings from this study indicate that resistance rates are particularly higher in intensive care units and that increasing resistance rates to antibiotics can affect empirical treatment choices for urinary tract infections (UTIs). This underscores the necessity for careful use of antibiotics and regular monitoring of current resistance profiles.

AUTHOR CONTRIBUTIONS

Hypotesis: A.Ç., S.Z.Ö., M.D., Ç.E., E.M., İ.K.; Design: A.Ç., Ç.E; Literature review: A.Ç., S.Z.Ö., M.D., Ç.E., E.M., İ.K.; Data Collection: A.Ç., S.Z.Ö; Analysis and/or interpretation: A.Ç., S.Z.Ö., M.D., Ç.E.; Manuscript writing: A.Ç

CONFLICT OF INTEREST

In the conflict of interest section, if there is no conflict of interest, the “Authors declare that there is no conflict of interest.” statement should be included.

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Research Article

Melatonin Extraction and Determination by UHPLC-FD from Different Plants

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ABSTRACT: Many plants have become a piece of the traditional food as human diet and evaluated in folk medicine to treat diseases. They are very rich in terms of nutraceuticals. Melatonin functions as a free radical scavenger and controls the regulation of the sleep–wake cycle in mammals. In this study, we analyzed melatonin content in the herba parts of *Stachys lavandulifolia*, *Tribulus terrestris*, and *Helichrysum arenarium,* and unripe fruits of *Prunus armeniaca* L., *Prunus cerasifera*, and *Prunus amygdalus* L. A solvent mixture of methanol:ultrapure water:HCl (70:29.9:01 v/v/v) was used for the extraction of melatonin from plant samples. The determination of melatonin in the extracts was carried out by high pressure liquid chromatography with fluorescence detector (UHPLC-FD). Melatonin concentration was 0.0231 mg/kg for herba of *Helichrysum arenarium* and 0.0018 mg/kg for *Prunus armeniaca* L. Melatonin was not detected in other plant samples.

Keywords: *Helianthus annuus, Prunus armeniaca L., Melatonin*

1 INTRODUCTION

Melatonin, a hormone primarily known to its role in regulating the sleep-wake cycle in humans, has emerged as a fascinating compound found not only in animals but also abundantly in various plants and fruits. This naturally occurring substance has garnered considerable interest due to its potential health benefits beyond sleep regulation. Understanding the presence and significance of melatonin in medicinal plants and selected fruits provides insights into its broader physiological roles and therapeutic potential.

In recent years, research has increasingly focused on identifying melatonin in botanical sources, revealing a diverse array of plant species that produce this hormone. This discovery underlines that melatonin is not only an endogenous mammalian hormone and its importance in plant physiology and ecology.

Melatonin (N-acetyl-5-methoxytryptamine) is a naturally occurring indoleamine hormone produced within the body. In mammals, melatonin serves as a biological regulator

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affecting various functions, including mood, sleep, hormone regulation, immune responses, circadian rhythms, and sexual behavior [1]. Identified in plants in 1995, melatonin also plays a role in processes such as photoperiodicity, flowering, and growth [2]. Additionally, it acts as a powerful antioxidant by neutralizing free radicals and enhancing antioxidant enzyme activity [3]. Research into melatonin's therapeutic benefits has revealed its wide range of bioactivities, including anticancer effects, anti-inflammatory properties, cardiovascular protection, immune system support, antidiabetic effects, neuroprotection, anti-aging benefits, and overall antioxidant activity [4–6]. Studies suggest that consuming melatonin-rich foods can elevate serum melatonin levels and improve antioxidant capacity in humans [7].

The use of plants dates back to ancient times, long before the advent of agriculture [8], and provides numerous advantages and opportunities for societies [9]. Many studies have shown the presence of melatonin in different plant species and tissues such as *Lupinus albus* L., *Hordeum vulgare* L. [10], *Prunus cerasus* [11], *Nicotiana tabacum* [12], *Hypericum perforatum* [13], *Helianthus annuus*, *Brassica nigra*, *Apium graveolens* [14], *Lycium barbarum*, and *Morus rubra* [15]. But the melatonin content of many plants is still unknown. In this study, the melatonin content of the herbal parts of three herbaceous plants (*Stachys lavandulifolia*, *Tribulus terrestris*, and *Helichrysum arenarium*) and unripe fruits of three stone fruit species (*Prunus armeniaca* L., *Prunus cerasifera*, and *Prunus amygdalus* L.) were investigated.

2. MATERIAL AND METHOD

2.1 Chemicals

The solvents methanol, ethanol, acetonitrile, and formic acid were supplied from Merck (Darmstadt, Germany) and were of HPLC analytical grade. Sodium acetate (≥99% purity) and melatonin (pure reagent) from Merck (Darmstadt, Germany) were purchased. Ultrapure water was prepared by a Millipore Direct-Q 3 UV-R water purification system (Molsheim, France).

2.2 Plant Material

In this study, *Stachys lavandulifolia*, *Tribulus terrestris*, and *Helichrysum arenarium* consumed as tea and *Prunus armeniaca* L., *Prunus cerasifera*, and *Prunus amygdalus* L. from stone fruits were used as plant materials.

Stachys lavandulifolia is known for its strong anti-oxidant effect, mild calming, and appetizing tea. This herb is also used to reduce anxiety and cure gastrointestinal

disorders [16-18]. *Tribulus terrestris* has been used in folk medicine throughout history to treat disorders such as impotence, rheumatism, edema, hypertension, and kidney stones [19]. *Helichrysum arenarium* is an herb known for its cholagogue, choleretic, hepatoprotective, and detoxifying properties, which have an important place in the traditional medical practice of Europe. The plant supports liver health by helping to remove harmful substances from the body and regulates the digestive system [20]. *Prunus armeniaca* is used for different purposes in various cultural and medical traditions. In particular, apricot seeds and oils are used to treat gynecological diseases, rheumatic pains, headaches, and skin hyperpigmentation. It is also used as a decoction for diseases like asthma, cough with phlegm, and fever. In China and other Asian countries, it is used in traditional medicines for viral infections and respiratory problems [21]. *Prunus cerasifera* fruit is rich in vitamins A, B1, B2, and C and sugar and can be used in diets. It is good for skin, hair, and eye health, besides, fresh plums are a good tonic for the kidney and stomach and are good for arthritis and rheumatism. In modern medicine, dried plums are powdered and used for coughs, colds, and sore throats. The oil obtained from the seeds of some plum varieties is very good for the beauty of the skin [22]. *Prunus amygdalus* is considered nutritious as it is a rich source of fat and protein. As part of its nutritional importance, it has also been reported to have beneficial effects on cholesterol levels and lipoprotein profiles in human blood, particularly lowering low-density lipoprotein (LDL) cholesterol [23-25].

Fruit samples from a farmer's garden in Malatya and other plant samples from a spice shop in Bingöl were obtained.

2.3 Extraction Procedure

The homogenized plant samples were extracted in the solvent mixture of methanol: ultrapure water: HCI (70:29.9:0.1 v/v/v). The samples (10.00 g) was extracted with methanol:ultrapure water:HCI (100 mL) on a ultrasonic bath for 1 hours and 37°C. The obtained extracts were concentrated with the help of rotavapor under low pressure and 40 °C. The resulting dry extract was dissolved in 5 mL of 90% methanol and then was filtered through a 0.45 µm PVDF (polyvinylidene difluoride) filter. This supernatant was used in analyses of melatonin.

2.4 UHPLC-FD analysis of melatonin

Chromatographic studies were quantified by UHPLC (Shimadzu Technologies, Kyoto, Japan), equipped with a RF-20 A

model fluorescence detector (FD). Analytical separations were performed using a Welch Welchrom C18 5 mm reversed-phase column (250 mm×4.6 mm). Isocratic elution was performed with 1 mL/min flow rate at 25°C, and the injection volume was 20 µL. The solvent mixture of methanol: water: formic acid (55:44.9:0.1 v/v/v) was used as the mobile phase, and the fluorescence detector were measured at the excitation/ emission wavelengths 275/345 nm [26]. The analytical parameters for melatonin are given in Table 1.

Table 1. Analytical parameters for melatonin analysis

Compound	Retention time	Linear equation	Linear range (mg/L)	R^2
Melatonin	5.134	y=799.62x+68.507	0.01-0.5	0.9996

3. RESULT

In the present study, it was investigated the melatonin content of the herba parts of *Stachys lavandulifolia*, *Tribulus terrestris*, and *Helichrysum arenarium,* and the unripe fruits of *Prunus armeniaca* L., *Prunus cerasifera*, and *Prunus amygdalus* L. To quantify melatonin content, the melatonin standart curve was established using the UHPLC-FD system within the range of 0.01-0.5 mg/L (Figure 1), and the results expressed as mg/kg.

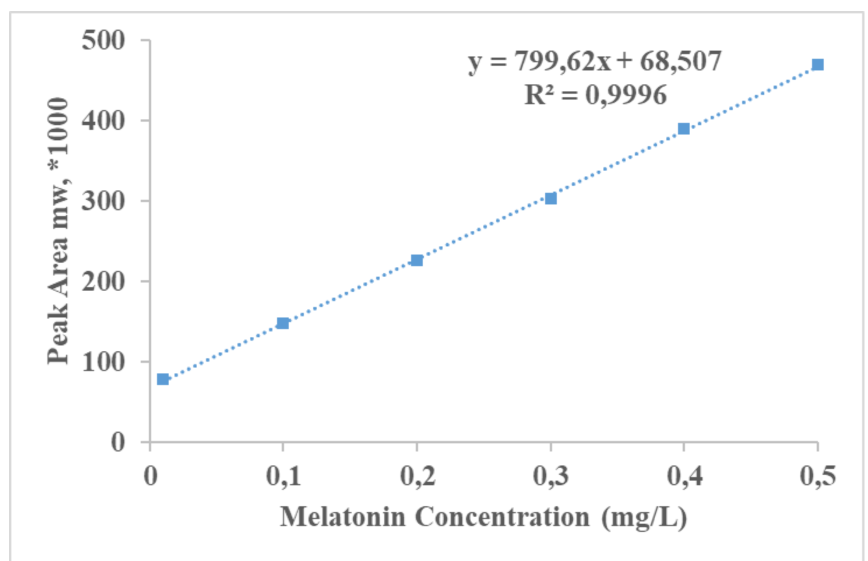

Figure 1. Calibration curve for the melatonin.

Melatonin concentration for plant samples was calculated based on the UHPLC peak areas. Melatonin results were presented in Table 2. Between herbaceous plants, melatonin was detected only in *Helichrysum arenarium*, while melatonin wasn't detected in *Stachys lavandulifolia* and *Tribulus terrestris.* Similarly, melatonin was detected only in *Prunus armeniaca* L. among stone fruit species, whereas it was not detected in *Prunus cerasifera* and *Prunus amygdalus* L.

Table 2. Melatonin content of plant samples (mg/kg)

Plant Samples (Herba)	Melatonin Content	Plant Samples (Unripe Fruit)	Melatonin Content
Stachys lavandulifolia	ND	*Prunus armeniaca* L.	0.0018±0.00
Tribulus terrestris	ND	*Prunus cerasifera*	ND
Helichrysum arenarium	0.0231±0.002	*Prunus amygdalus* L.	ND

ND: Not Dedection

4 DISCUSSION

In recent years, there has been increased interest in determining the roles of plant-derived compounds in improving human health. Plants are splendid sources of bioactive compounds and nutrients. Epidemiological studies have emphasized the importance of consuming edible plants on the prevention of many human diseases [27]. In mammals, melatonin acts as a biological regulator affecting numerous processes, including immune responses, mood, circadian rhythms, hormone regulation, sexual behavior, and sleep. This natural hormone offers several health benefits, such as enhancing antioxidant enzyme activity and serving as a strong antioxidant that neutralizes free radicals. In plants, melatonin plays roles in flowering, photoperiodicity, growth, development, and boosting resilience to environmental stressors [28-30]. In the present study, the melatonin content was examined in the herba parts of *Helichrysum arenarium, Stachys lavandulifolia, Tribulus terrestris,* and unripe fruits of *Prunus armeniaca* L., *Prunus cerasifera,* and *Prunus amygdalus* L. Different amounts of melatonin were detected in *Helichrysum arenarium* and *Prunus armeniaca* L. plant samples. However, no melatonin was detected in other plant samples. Melatonin is a naturally occurring compound found in small quantities in various foods, including edible plants. Researchers have detected melatonin in different plant parts such as flowers, fruits, stems, roots, leaves, and seeds. For instance, Chen et al. [31]

reported that the melatonin concentration in dried goji berries and white mulberry leaves was measured at 530 and 1510 ng/g, respectively. In a study conducted by Kolar and Malbeck [32], it was reported that the melatonin amount in blackberry was measured as 21 pg/g. In *Prunus* species, it was reported that melatonin content in ripe fruits of *Prunus cerasus* and *Prunus avium* species ranged from 2.06 to 13.46 ng/g [33, 34] and 0.01–20 ng/g [33-35], respectively. Variations of the melatonin contents in different plants are reasonable relative to the physical and chemical characteristics of plant samples. The melatonin concentrations in plants are closely influenced by factors such as extraction methods, species, harvesting time, growing environment, varieties, and cultivated methods. Regarding the melatonin content of plants, considerable differences exist in the course of developmental processes, too. The various environmental or biotic stress factors lead to changes in melatonin. In conclusion, the identification of biologically active molecules in edible vegetables and fruits, and herbaceous plants used in traditional medicine will shed light on new studies.

5 AUTHOR CONTRIBUTIONS

Hypotesis: S.E., Y.U.; Design: E.Y., A.M., S.E.; Literature review: Y.G., A.T.T.; Data Collection: Y.G., A.T.T, A.M., E.Y; Analysis and/or interpretation: Y.U., Y.G., A.T.T., A.M., E.Y., Z.M.; Manuscript writing: Y.U., S.E.

6 CONFLICT OF INTEREST

Authors declare that there is no conflict of interest. Jsldbfkjs

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