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ORIGINAL ARTICLE / ÖZGÜN MAKALE

THE COMPARISON OF ANTIOXIDANT AND ANTIBACTERIAL ACTIVITY OF NOVEL PYRIMIDO[1,2-a]PYRIMIDINE COMPOUNDS

YENİ PİRİMİDO[1,2-a]PİRİMİDİN BİLEŞİKLERİNİN ANTİOKSİDAN VE ANTİBAKTERİYAL ÖZELLİKLERİNİN KARŞILAŞTIRILMASI

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ABSTRACT

Objective: *Heterocycles with a pyrimidopyrimidine scaffold have been the focus of interest of researchers in the field of medicinal chemistry as they have a wide range of biological properties. In the current study antioxidant capacity and antibacterial activity of a series of new pyrimido[1,2-a]pyrimidines were investigated*

Material and Method: *All these novel compounds were screened for their in vitro antioxidant effectiveness using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging test and the antibacterial activity was determined using the broth microdilution method according to The European Committee on Antimicrobial Susceptibility Testing standards (EUCAST) as a minimal inhibition concentration (MIK) against some Gram- positive and negative ATCC strains.*

Result and Discussion: *The overall range of DPPH free radical scavenging activity was found to be 3.86-6.90% at 0.1mM and 6.28–16.59% at 1mM for the novel pyrimidopyrimidine derivates. Since the free radical scavenging activities of all the compounds were below 20%, it was considered that the weak activity of compounds might be due to the absence of an enolizable amide group in the pyrimidine ring. In addition to that, some of the selected compounds showed weak antioxidant activity. MIC values of all compounds were found as > 100 µg/ml against Staphylococcus aureus ATCC 29213, methicillin resistant Staphylococcus aureus ATCC 43300, Enterococcus faecalis 29212, Escherichia coli ATCC 25922, Klebsiella pneumoniae ATCC 13883, Pseudomonas aeruginosa ATCC 27853. The data obtained from present study require the synthesis of derivatives*

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with various substituents in different positions of the pyrimidine ring in order to be able to evaluate and interpret more comprehensively in future studies.
Keywords: *Antibacterial activity, antioxidant activity, MIC, pyrimidopyrimidine, radical scavenging*

ÖZ

Amaç: *Pirimidopirimidin yapı iskeletine sahip heterohalkalı bileşikler, sahip oldukları çok çeşitli biyolojik özellikleri sayesinde tıbbi kimya alanındaki araştırmacıların ilgi odağı olmuştur. Bu çalışma ile, bir seri yeni pirimido[1,2-a]pirimidin türevlerinin antioksidan kapasitesi ve antibakteriyel aktivitesi araştırılmıştır.*
Gereç ve Yöntem: *Sentezlenen tüm bileşiklerin in vitro antioksidan aktiviteleri 2,2-difenil-1-pikrilhidrazil (DPPH) radikal süpürme testi kullanılarak gerçekleştirilmiştir. Antibakteriyel aktivite çalışmaları, bazı Gram-pozitif ve negatif ATCC suşlarına karşı minimum inhibisyon konsantrasyonu (MİK) olarak Avrupa Antimikrobiyal Duyarlılık Testi standartlarına göre sıvı mikrodilüsyon yöntemi kullanılarak belirlenmiştir.*
Sonuç ve Tartışma: *Yeni pirimidoprimidin türevleri için DPPH serbest radikal süpürme aktivitesinin genel aralığının 0.1 mM'de %3.86-6.90 ve 1 mM'de %6.28-16.59 olduğu bulunmuştur. Tüm bileşiklerin serbest radikal süpürme aktiviteleri %20'nin altında bulunduğundan, pirimidin halkasında enolize olabilen bir amit grubunun bulunmamasından kaynaklı olarak bileşiklerin aktivitesinin zayıf olduğu düşünülmüştür. Buna ek olarak, seçilen bileşiklerin bazıları zayıf antioksidan aktivite göstermiştir.. Tüm bileşiklerin MİK değerleri, Staphylococcus aureus ATCC 29213, metisiline dirençli Staphylococcus aureus ATCC 43300, Enterococcus faecalis 29212, Escherichia coli ATCC 25922, Klebsiella pneumoniae ATCC 13883, Pseudomonas 27853 ATCC'ye karşı > 100 µg/ml bulunmuştur. Bu çalışmadan elde edilen veriler, ileriki çalışmalarda daha kapsamlı değerlendirme ve yorumlama yapabilmek amacıyla pirimidin halkasının farklı konumlarında çeşitli sübstitüentlerin bulunduğu türevlerin sentezini gerektirmektedir.*
Anahtar Kelimeler: *Antibakteriyel etki, antioksidan etki, MİK, pirimidopirimidin, radikal süpürücü*

INTRODUCTION

Pyrimidines were concerned about effectiveness in biological processes with their similarity to natural pyrimidine bases (uracil, cytosine, thymine, adenine, guanine) [1-3]. Pyrimidine compounds with their bicyclic and tricyclic derivatives are commonly studied scaffolds in medicinal chemistry because of their large spectrum of bioactivities [4-6]. Since it has been very well known that various diseases such as diabetes, stroke, and inflammation, originated from oxidative stress and its harmful effects development of compounds inhibiting oxidative stress might be considered as an efficient approach for treatment of many diseases [7-9]. Pyrimidopyrimidines are known as a specific class of fused ring-heterocycles having nitrogen heteroatoms. Pyrimidopyrimidines and their known four structural isomers have deserved interest for their biological and pharmacological activities, especially anticancer, antibacterial, antiviral and antioxidant activity. Among these, pyrimido[4,5-d]pyrimidine and pyrimido[5,4-d]pyrimidine have been used as precursors in drug research (such as dipyridamole Figure 1) owing to the existence of several simple reported methods for these compounds.

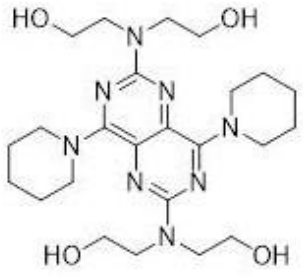

Figure 1. Structure of dipyridamole

Pyrimido[1,2-a]pyrimidine ring system is thought to have great similarity to ring systems of quinolone antibiotics; such as naphtalene, quinoline and naphtyridine so that new derivatives of pyrimido[1,2-a]pyrimidines with similar functional groups may produce potential antibacterial compounds. Quinolones are broad-spectrum, antibacterials that target the bacterial enzymes; topoisomerase II and topoisomerase IV [10]. On this basis, pyrimidopyrimidines could be derivatized with functional groups similar to quinolones and expected to show desired antibacterial effects (Figure 2) [11]. Pyrimido[1,2-a]pyrimidines are one of the most important members of this family. But existing studies on these compounds have not received sufficient attention. A couple of applicable synthetic methods are described in the literature for pyrimido[1,2-a]pyrimidines, but nearly all of them have been limited to only a few molecules [12-14].

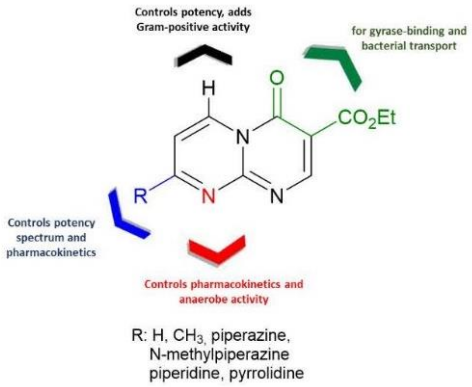

Figure 2. Design strategy of pyrimido[1,2-a]pyrimidines

Our previous study provided a new approach for the synthesis of novel pyrimido[1,2-a]pyrimidines in high yields by reactions of 2-aminopyrimidines with diethyl ethoxymethylenemalonate (EMME) using microwave irradiation and conventional heating and published elsewhere [15]. For this purpose, in present research, it was firstly aimed to synthesize novel pyrimido[1,2-a]pyrimidines and investigate their antibacterial activity by broth microdilution method in agreement with European Committee on Antimicrobial Susceptibility Testing (EUCAST) standards [16] and antioxidant activity with the use of 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay [17,18], which are hitherto unknown in the literature. The novel derivatives were characterized by spectral data together with elemental analysis and were used for their antioxidant and antibacterial screening. Achieving the expected antibacterial and antioxidant effect will provide an alternative to the development of potential antibacterials that can be used for therapeutic purposes and which in turn might also pose antioxidant properties as well.

MATERIAL AND METHOD

Synthesis

A series of novel pyrimido[1,2-a]pyrimidine derivatives containing electron-withdrawing and electron-donating substituents on the ring have been successfully synthesized. All spectral analyzes and characterizations were performed and published in a previous study [15].

Antioxidant capacity determination of newly synthesized ethyl pyrimido[1,2-a]pyrimidine-3-carboxylate derivatives (3 a-g)

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The free radical scavenging activity of the newly synthesized compounds was evaluated by an adaptation of previously reported protocols with slight modifications by their capability to bleach the stable radical DPPH [17,18]. Test compounds (0.1mM and 1mM) were incubated with DPPH solution (150 μM) at room temperature for 30 min. The value of 517 nm was measured as the absorbance against

a blank using a UV-Vis spectrophotometer. All experiments were carried out in triplicate. Ascorbic acid was used as a reference compound. The radical scavenging activities of the compounds were expressed as the percent of inhibition and calculated using the equation below:

$$\text{Radical scavenging activity (\%)} = [(A0-A1)/A0]*100$$

Where A0 is the absorbance of the control reaction and A1 is the absorbance in the presence of the test compounds or standard.

Antibacterial activity studies

Antibacterial activity of 7 different quinolone-like compounds was tested on *S. aureus* ATCC 29213, *E. faecalis* ATCC 29212, *K. pneumoniae* ATCC 13883, *P. aeruginosa* ATCC 27853, and *E. coli* ATCC 25922. Antibacterial activity test was evaluated as a minimal inhibition concentration (MIC) in agreement with EUCAST (EUCAST, 2022b) standarts by broth microdilution method. The method was performed with serial dilution of compounds in cation adjusted Mueller Hinton Broth using sterile, U bottom, 96 well plates. The bacterial suspension was obtained by 1:100 dilution of the suspension prepared from fresh bacterial culture to 0.5 Mcfarland turbidity. 100 µl bacterial suspension was added into the wells. and incubated at 35±2°C for 16 to 20 h. The minimum concentration that inhibited the bacterial growth was accepted as MIC. The tested compounds were dissolved in dimethyl sulfoxide (DMSO). The effectiveness of DMSO was also tested and ciprofloxacin was used as a positive control.

RESULT AND DISCUSSION

Synthesis of pyrimido[1,2-a]pyrimidines

In our previous study, various experimental conditions such as different solvents, reaction temperatures, and also the molar ratio of reagents had been examined in detail both for the conventional heating and microwave irradiation to find the optimum conditions for the synthesis of pyrimido[1,2-a]pyrimidines (3a-g). In all cases, the best solvent was chosen as diphenyl ether owe to its inert nature. The optimum temperature interval was determined as 200-220°C degrees. Above this temperature decomposition reactions occurred, and tar-like masses were observed. When the temperature was held under this temperature range, only mono-adduct enamine was obtained. Heating strategies were also compared and it was observed that microwave-assisted synthesis was the most applicable method for this type of cyclization reaction. The basic synthetic process consists of two steps; syntheses of 4-substituted 2-aminopyrimidines and then cyclization of these 2-aminopyrimidines with EMME in one-step under optimal conditions. Consequently, pyrimido[1,2-a]pyrimidines derivatives bearing one substituent (CH_3, pyrrolidinyl, piperidinyl and/or morpholinyl) at the 8- position have been synthesized with good yields Figure 3 [15].

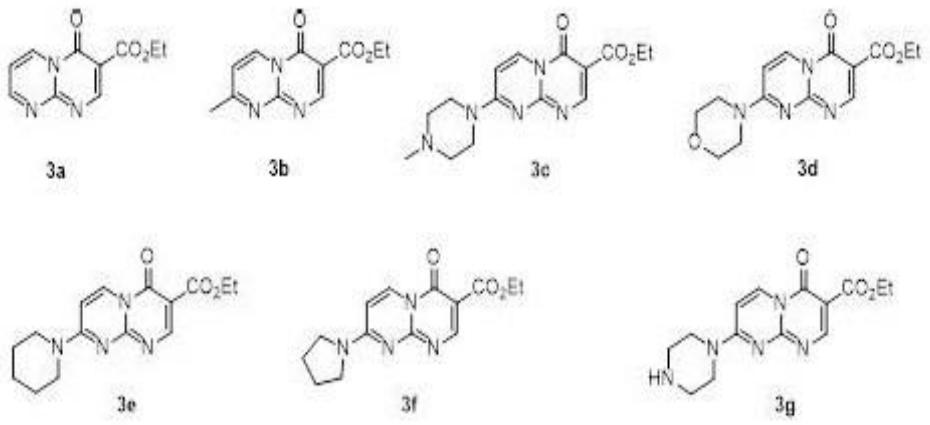

Figure 3. Synthesized pyrimido[1,2-a]pyrimidines

Antioxidant activity:

In vitro antioxidant activities of the novel pyrimido[1,2-a]pyrimidines were determined using DPPH radical scavenging tests, as shown in Figure 4 and Table 1. The results obtained were compared with standard ascorbic acid antioxidant activity. A major part of the new compounds exhibited low to moderate interaction with the DPPH radical at 0.1 mM and 1.0 mM concentrations compared to ascorbic acid as standard. Maximum DPPH radical scavenging activity was observed in the synthetic compounds.

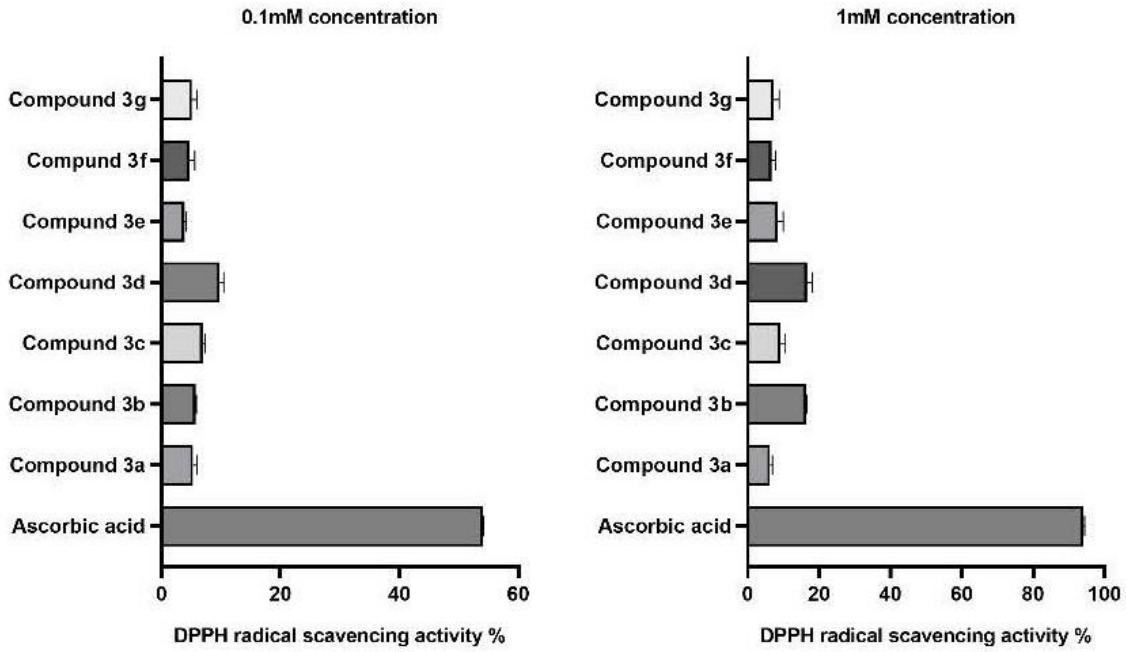

Figure 4. (A) DPPH radical scavenging activity (%) of the compounds at 0.1mM (B) DPPH radical scavenging activity (%) of the compounds at 1mM. The DPPH scavenging activity of ascorbic acid (reference compound) was used for comparison and is shown in the graphs

Table 1. DPPH radical scavencing activies of the compounds

Compounds	DPPH free radical scavenging activity (%)	
	0.1mM	**1mM**
3a	5.27±0.69	6.28±0.62
3b	5.75±0.11	16.27±0.92
3c	6.90±0.37	9.11±1.51
3d	9.80±0.62	16.59±1.50
3e	3.86±0.32	8.29±1.63
3f	4.61±0.96	6.69±1.10
3g	5.06±0.91	7.36±1.44
Ascorbic acid	54.02±0.11	93.93±0.48

Values are expressed as means ± standard deviation (S.D.) of three different experiments.

Radical scavenging activities of all compounds were designated by the interacting capability of compounds with DPPH as stable free radicals. Compound 3a, having no substituent on the pyrimido[1,2-a]pyrimidine, was chosen as a model compound for evaluating its DPPH radical scavenging activity. Compound 3a has been tested in 0.1 mM and 1mM concentrations for radical scavenging activity. All compounds in these series were screened for their antioxidant activity in both concentrations mentioned above. The antioxidant activities were expressed in Table 1 and the results were compared with ascorbic acid. The results shown in Table 1 reveal that all compounds have weak antioxidant activity compared to ascorbic acid. The overall range of DPPH radical scavenging activity was found to be 3.86-6.90 at

0.1mM and 6.28–16.59 at 1mM. It can be seen from the results that free radical scavenging activities of all the compounds were below 20%. It was considered that the weak activity of compounds might be due to the absence of an enolizable amide group in the pyrimidine ring. According to current results maximum DPPH radical scavenging activity at 1 mM was obtained in compounds 3b and 3d, which possess a methyl and a morpholinyl group at the 8- position of the pyrimido[1,2-a]pyrimidine ring. Both radical scavenging activities of 3b and 3d were weaker than the standard ascorbic acid, so they could be considered as a moderate reducing agent.

Antibacterial activity

The MIC values of all compounds tested were found higher than 100 µg/ml against *Staphylococcus aureus* ATCC 29213, methicillin resistant *Staphylococcus aureus* ATCC 43300, *Enterococcus faecalis* 29212, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, and *Pseudomonas aeruginosa* ATCC 278535922. The results were given in Table 2. Considering the activity of the synthesized compounds, it is thought that the compounds may have an antibacterial activity above the tested concentrations. Since there is no publication about present test compounds in the literature, a comparison could not be made. However, when the results are evaluated according to EUCAST standards, the effective concentration of quinolone antibiotics currently in use, such as ciprofloxacin, varies according to bacteria species but is quite low, and the results obtained from the control strains in this study are listed in Table 2. Therefore, an effect that can be detected at a concentration above 100 µg/ml was not tested in the present study, as it would not be considered as effective compared to the standards and literature examples [19].

Table 2. Antibacterial activity results for tested compounds as MIC

Compounds	Minimal inhibition concentrations (µg/ml)					
	Staphylococcus aureus **ATCC 29213**	**Methicillin resistant** ***Staphylococcus aureus*** **ATCC 43300**	***Enterococcus faecalis*** **ATCC 29212**	***Klebsiella pneumoniae*** **ATCC 13883**	***Pseudomonas aeruginosa*** **ATCC 27853**	***Escherichia coli*** **ATCC 25922**
3-a, 3-b, 3-c, 3-d, 3-e, 3-f, 3-g	> 100	> 100	> 100	> 100	> 100	> 100
Ciprofloxacin	0.25	0.5	0.5	0.0625	0.25	0.0078

It is thought that this may be due to the absence of fluorine atoms in the ester forms of the compounds. Fluoroquinolones, such as ciprofloxacin that used as control in present study, show their mechansim of action by inhibiting DNA topoisomerases of most Gram-negative and Gram-positive bacteria and act at low concentrations [20], for example, ciprofloxacin MIC value were differs between 0.004-0.016 mg/ml for *E. coli* ATCC 25922 [21]. The current study is important as it will provide data to the literature in terms of antibacterial test results.

Due to their privileged biological properties, heterocycles with pyrimidopyrimidine scaffolds have been the focus of the attention of researchers. These heterocycles are used as antibacterial agents, cancer cell growth inhibitors, antioxidants, antiviral agents, and antitumor agents [22-25]. Therefore, the current study aimed the investigate the new series of pyrimido[1,2-a]pyrimidine derivatives for their antibacterial and antioxidant potentials. Overall, the free radical scavenging abilities of all synthesized compounds were considered with the stable DPPH radical methods. Antibacterial activity of all compounds against some Gram-positive and Gram-negative bacteria was tested and it was determined that test compounds could show dose-dependent activity. It was also evaluated that a number of the selected compounds showed weak antioxidant activity. Therefore, this preparatory study can be considered promising research to further investigate their broad spectrum of pharmacological activities. The previous studies which performed on pyrimido[1,6-a]pyrimidine, pyrimido[1,6-c]pyrimidine and pyrido[2,3-d]pyrimidine skeletons provided efficient biological activites [26, 27]. Therefore, since the excepted biological activities could not be observed with the present substitutions of the pyrimido[1,2-a]pyrimidine ring, the synthesis of various substituents from different positions of the pyrimidine scaffold is required for further evaluation in future studies.

AUTHOR CONTRIBUTIONS

Concept: D.B.; Design: D.B., M.D.Ö., M.E.K.; Control: D.B.; Sources: D.B., M.D.Ö., M.E.K.; Materials: D.B., M.D.Ö., M.E.K.; Data Collection and/or Processing: D.B.; Analysis and/or Interpretation: M.D.Ö., M.E.K.; Literature Review: D.B., M.D.Ö., M.E.K.; Manuscript Writing: D.B.; Critical Review: D.B., M.D.Ö., M.E.K.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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