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The Compound Specific Antibacterial Activities of Major Urolithins and Their Methyl Ethers

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Abstract: The investigation of biological activities of natural products, particularly considering the secondary metabolites, continuously receives attention. Urolithins, the bioavailable metabolites of ellagitannins, were shown to possess enzyme inhibitor, antioxidant, and anti-inflammatory compounds in scientific studies conducted in the last two decades. Regarding the limited number of studies related to their antimicrobial activity, this study aimed to synthesize major urolithins (Urolithin A and B) concomitant to their methyl ether derivatives and screen their antibacterial activity against some Gram positive and Gram negative bacteria. In parallel to the antibacterial activity, the synergistic and antagonist properties of the compounds were also analyzed in the presence of reference beta-lactam antibiotics. The results displayed the improvable characteristics of urolithin scaffold to be employed in antibiotic drug design studies. In addition, the antagonist effect of some compounds on the antibacterial action of standard molecules also pointed out the compound specific activities of the title molecules.

Keywords: Urolithin, synthesis, antibacterial activity, antagonism, synergism.

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INTRODUCTION

Natural compounds, generally referred to as secondary metabolites of living things, have always attracted the curiosity of scientists to discover alternative strategies for the treatment of many states of disease (1,2). Indeed, there are many natural product-based drugs still used today. This type of research studies also guide to understand the possible biological activities of secondary metabolites, particularly in case of routine exposure to them via different sources (2,3). For instance, papaver somniferum alkaloids (e.g., morphine, codeine) have been used in different preparations either for treatment of pain or abuse purposes for centuries, however, the motivation on research for natural product chemistry, and biological activity screening of natural products have led to the discovery of opioid receptors and synthetic opioid molecules throughout the 20th century (4). As another example, the work of Alexander Fleming on the discovery of beta-lactam antibiotics from mold still has life-saving effects in the treatment of many life-threatening infectious diseases (5).

Urolithins, the hydroxyl substituted benzo[c] chromen-6-one derivatives, have attracted attention as natural compounds in the last two decades. In fact, these compounds are metabolism products in many mammalian species following exposure to ellagitannins (6,7). Nuts, berries, and particularly pomegranate are rich sources of ellagitannins. Many mammalians like humans regularly eat these diets. As seen in Figure 1, ellagitannins, the ester bond connected gallic acid derivative macromolecules, are subject to the gastrointestinal system microflora-catalyzed biotransformation reactions to yield out urolithin molecules, mainly as mono-, di-, tri-, and tetra-hydroxy substituted benzo [c] chromen-6-ones (8). The metabolism studies indicated that ellagitannins and their metabolism precursor molecule ellagic acid have negligible absorption from the gastrointestinal tract (9). However, the urolithins are bioavailable compounds. Indeed, urolithins appear in systemic circulation in two to three hours following the oral exposure to ellagitannin rich diet, particularly pomegranate (10).

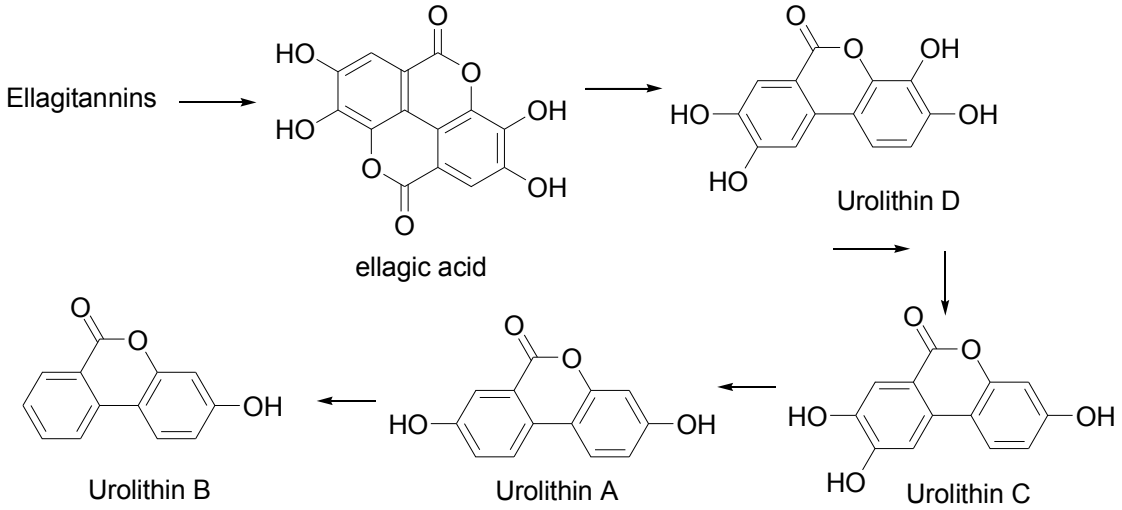

Figure 1: The formation of major urolithins, Urolithin A and B, through metabolism.

So far, many biological activities of ellagitannins have been shown under *in vivo* conditions (11). These were attributed to the urolithins, since a systemic effect can be seen only for bioavailable compounds. Among these activities, the antimicrobial activity gathered limited attention, since the main focus has been provided on ellagitannins (12). In one study, it was shown that urolithins A and B displayed antibacterial effects in the colon against *Yersinia enterocolitica* (13).

From this perspective, within this study, we have aimed to synthesize major urolithins (Urolithin A and B) and their methyl ether metabolites, formed through the catechol-O-methyl transferase activity. The antibacterial activities of the title compounds have been planned to be screened against several bacterial strains [i.e., *Escherichia coli*, *Staphylococcus aureus*, *Enterococcus faecalis*, and methicillin resistant *Staphylococcus aureus* (MRSA)]. Besides, the antagonist or synergistic activities of the compounds with a beta-lactam antibiotic have been aimed to be analyzed. To our knowledge, this has been the first study conducted on the total evaluation of urolithins in terms of their antibacterial activities in different Gram(+) and Gram(−) bacteria.

EXPERIMENTAL SECTION

Materials and instruments

All the chemicals and biologicals were purchased from local chemical suppliers of Turkish Republic of Northern Cyprus. They were used without purification unless otherwise stated. Thin layer chromatography studies were performed using Merck aluminum-packed silica gel plates to monitor the reactions. Ethyl acetate – n-hexane (1:1, v/v) was used as the mobile phase. Infrared spectra were obtained through a Shimadzu FT-IR Prestige spectrometer. Proton and carbon 13 NMR spectra of the title compounds were obtained via a Bruker-400 NMR spectrometer. Tetramethylsilane (TMS) was used as internal standard and deuterated dimethylsulfoxide (DMSO-d_6) was employed to dissolve the samples. The chemical shifts were reported in ppm. A Thermo Fisher Flash Smart CHNS elemental analyzer was employed for elemental analysis.

Chemistry

General synthesis protocols

Previously known procedures were followed for the synthesis of the title molecules (Figure 2) (14). Accordingly, the hydroxy substituted urolithin analogues (i.e., Urolithin A and B), and 3-hydroxy-8-methoxy-6H-benzo[c]chromen-6-one were

synthesized through reacting 15 mmol of resorcinol either with 5 mmol of 2-bromobenzoic acid (to obtain Urolithin B), or 5 mmol of 2-bromo-5-hydroxybenzoic acid (to obtain Urolithin A), or 5 mmol of 2-bromo-5-methoxybenzoic acid (to obtain 3-hydroxy-8-methoxy-6H-benzo[c]chromen-6-one) in 18 mmol NaOH dissolved distilled water. The mixtures were refluxed for 1 h and added 22% of $CuSO_4$ solution in 15 mL distilled water at the end of the time. The products precipitated were filtered off and washed with 0.01 N 50 mL hydrochloric acid solution.

The alternative methoxy-substituted analogues (i.e., methyl ether of Urolithin B, and dimethyl ether of Urolithin A) were synthesized respectively treating Urolithin B and A with methyl iodide. Briefly, 5 mmol of urolithin A or B was treated with 5.5 mmol of NaH in DMF. Following stirring at rt for 3 min, the solutions were added appropriate amount of methyl iodide (i.e., 5.5 mmol methyl iodide to obtain the methyl ether of Urolithin B, and 10.5 mmol methyl iodide to obtain the dimethyl ether of Urolithin A). After stirring at rt for 3h, the reaction mixtures were poured into 50 mL of distilled water. The mixture was extracted with 3 times of 30 mL of ethyl acetate. Following the evaporation of collected organic phases, the compounds were purified through column chromatography employing ethyl acetate – n-hexane (1:1) as the mobile phase. Spectral characterizations of the molecules have also been previously stated (14).

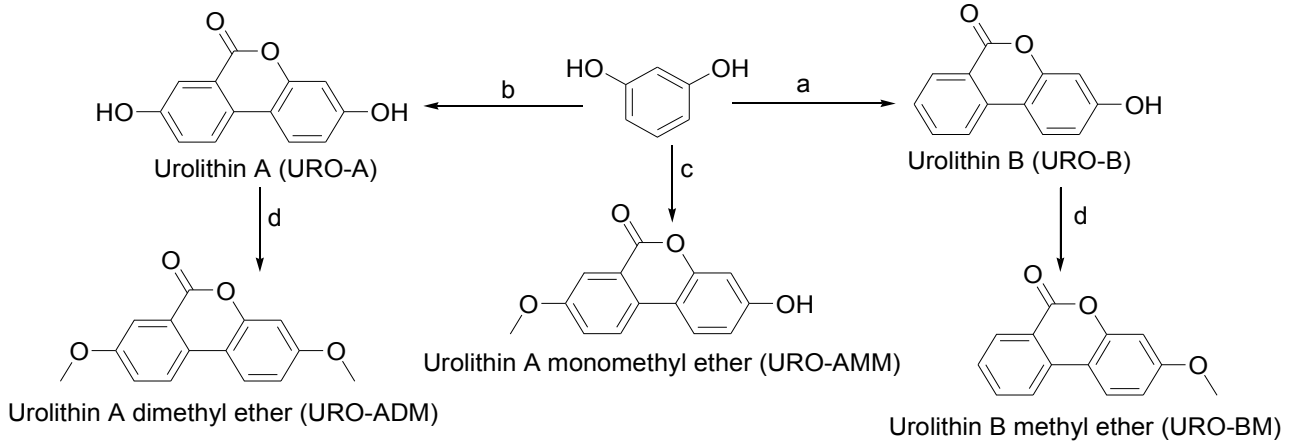

Figure 2: The synthetic protocol followed. a: 2-Bromobenzoic acid, NaOH, H_2O; b: 5-Hydroxy-2-bromobenzoic acid, NaOH, H_2O; c: 5-Methoxy-2-bromobenzoic acid, NaOH, H_2O; d: NaH, Methyl iodide, DMF

Antibacterial Activity

Bacterial strains

The antibacterial activity of the compounds was investigated against quality control strains of American Type Culture Collection (ATCC). *Staphylococcus aureus* ATCC 25923 (methicillin susceptible) and *Enterococcus faecalis* ATCC 29212 were used as representatives of Gram(+) whereas *Escherichia coli* ATCC 25922 was used as the representative of Gram(−) bacteria. MRSA strain that was isolated from the nose of a carrier and identified as *Staphylococcus aureus* by Gram characteristics, catalase and coagulase test was included in the study. The methicillin resistance of the strain was identified by disk diffusion method using cefoxitin disk (30 µg) as suggested by European Committee on Antimicrobial Susceptibility Testing (EUCAST) (15).

Bacteria were inoculated on Mueller Hinton agar (MHA) (Merck, Germany) by spread plate method and incubated at 37 °C for 24 hours under aerobic atmosphere. After incubation period, pure culture was derived by sub-culturing the single colony of each strain onto MHA and incubating under the same conditions mentioned above. Each of four strains was suspended in Mueller Hinton broth (MHB) and the suspensions were adjusted to the turbidity of 0.5 McFarland that are equivalent to the microorganism density of 1.5 x 10^8 cfu/mL. The inoculum of each strain was diluted using MHB to give an inoculum of 1 x 10^6 cfu/mL.

Preparation of the compounds

32.77 g/L of each of the compounds was prepared in dimethyl sulfoxide (DMSO), diluted 1:16 using MHB to obtain the concentration of 2048 mg/L and filtered using 0.45 µm pore sized syringe filters.

Determination of Minimum Inhibitory Concentrations (MICs)

The MICs of the compounds were investigated by broth microdilution method (16). Briefly, 50 µL of two fold diluted concentrations of the compounds were mixed in individual wells of 96 round bottom well plates with 50 µL of inoculum containing 1 x 10^6 cfu/mL of each strain. The final concentrations

of the compounds ranged from 1024 mg/L to 2 mg/L and the final concentration of DMSO in each well is ≤ 3%. For each run, a well containing 50 µL 3% DMSO with 50 µL inoculum of the respective strain was used as a positive control and a well including 50 µL 1024 mg/L with 50 µL MHB (instead of the bacterium) was used as a negative control. Ampicillin was used as internal control for *Enterococcus faecalis* ATCC 29212 and *Escherichia coli* ATCC 25922. On the other hand, penicillin G was used internal control for *Staphylococcus aureus* ATCC 25923 and MRSA. The microplates were incubated at 37 °C under aerobic atmosphere for 16 hours. MIC was regarded as the minimum concentration of the compound that inhibited the growth of the strain.

Effects of the compounds on MIC values of antibiotics

The effects of the compounds on ampicillin (Sigma-Aldrich) against *Escherichia coli* ATCC 25922 and *Enterococcus faecalis* ATCC 29212 and on penicillin G (Sigma-Aldrich) against *Staphylococcus aureus* ATCC 25923 and MRSA were investigated by broth microdilution checkerboard method (17). The compounds were prepared as described in the preparation of compounds and the antibiotics were prepared as suggested by the manufacturer. The final concentrations of the antibiotics ranged from eight times higher and sixteen times lower than expected MICs. The concentrations of the compounds eight times lower and higher than the MICs calculated by microdilution method were tested. 50 µL of the two fold increasing antibiotic concentrations was mixed with equal volume of two fold increasing concentrations of the compounds. The final organism concentration was 3 x 10^5 – 5x 10^5 cfu/mL in each well. The individual MICs of the antibiotics and the compounds were confirmed in the first row and column, respectively, of the microplate for each run. The plates were incubated under aerobic atmosphere at 37 °C for 16-20 hours.

For the combination of the compound with the antibiotic tested, summation of fractional inhibitory concentration (∑FIC) was calculated as the sum of FIC of compound and FIC of antibiotic formula; where FIC of a compound is the ratio of MIC of a compound in combination over the MIC of compound alone, and the FIC of an antibiotic is the MIC of antibiotic in combination divided by the MIC of the antibiotic alone. The interaction between the compound and the antibiotic was regarded as; Synergism, where ∑FIC ≤ 0.5, Indifference, where 0.5 < ∑FIC ≤ 4, and Antagonism, where ∑FIC >4.

RESULTS AND DISCUSSION

The antibacterial activities of the title compounds have been assessed against several Gram(+) and Gram(−) bacterial strains (i.e., *Escherichia coli* as Gram(−), and *Staphylococcus aureus*, *Enterococcus faecalis*, and MRSA as Gram(+) strains) and the MIC values measured are shown in Table 1. Accordingly, none of the urolithins displayed activity against the Gram(−) strain *Escherichia coli*. Beside the inactivity of URO-AMM, the rest four urolithin derivatives displayed some activity against MRSA. In addition, all the compounds displayed weak to moderate activity against *Enterococcus faecalis*. On the other hand, beside the weak activity of URO-A, none of the compounds was found to be active against *Staphylococcus aureus*. The MIC values of the title molecules were also found weaker in comparison to the activities of reference molecules, ampicillin and penicillin G, against the strains employed in the study. Among the compounds tested, URO-A has been found as the only molecule that displays activity against the strains tested. This outcome is significant considering the fact that URO-A is one of the major metabolites found in systemic circulation following exposure to ellagitannin rich diet (18). URO-B, another major metabolite, was found to be inactive in general, beside its activity against *Enterococcus faecalis*.

On one hand, the results obtained pointed out the significance of URO-A molecule to be employed in antibacterial drug design studies as a scaffold to be developed to obtain more active antibacterial compounds. On the other hand, as methyl ether analogs typically exhibited weaker activities, phenolic hydroxyls appear to be important substitutions for the antibacterial activities obtained.

Table 1: MIC values (mg / L) of the title urolithin derivatives.

Title compound	*E. coli*	*S. aureus*	*E. faecalis*	MRSA
URO-B	> 512	> 512	32	256
URO-BM	> 512	> 512	128	64
URO-A	> 512	64	32	64
URO-ADM	> 512	> 512	128	64
URO-AMM	> 512	> 512	128	> 512
Ampicillin	4	NT	0.5	NT
Penicillin G	NT	0.03	NT	8

NT: Not tested

One of the important research fields in the action of antibacterials is the investigation of the effect of the natural products on the antibacterial activity of known and used antimicrobial agents (19,20). From this perspective, the effect of title urolithin compounds on the antimicrobial action of reference molecules (i.e., ampicillin, penicillin G) against the bacterial strains employed was tested. The results obtained are shown in Table 2.

Accordingly, none of the compounds tested caused a change on the MIC of ampicillin over *Escherichia coli*. URO-AMM (i.e., the monomethyl ether of Urolithin A) increased the MIC of penicillin G against *Staphylococcus aureus* more than 4 fold. Therefore, its action was characterized as antagonist on the activity of penicillin G. Although URO-AMM doubled the MIC of penicillin G against MRSA, since ∑FIC was less than 4, the net effect was evaluated as indifferent.

One of the major urolithin metabolites, URO-B, also displayed considerable effects. At one hand, it lowered the MIC of ampicillin against *Enterococcus faecalis*. Since ∑FIC was not less than 0.5, the overall effect was assessed as indifference. However, URO-B increased the MIC of penicillin G for more than 4 fold against MRSA, and therefore, its activity was found to be antagonist for the activity of penicillin G over MRSA.

Besides, the URO-A, URO-BM, and URO-ADM have been found not to have any effect on the MIC values of ampicillin and penicillin on *Staphylococcus aureus* and *Enterococcus faecalis*. On the other hand, URO-BM and URO-ADM combination with penicillin G was found to have two times higher MICs than the MIC of penicillin G alone against MRSA. Since the ∑FIC was less than 4, these activities were evaluated as indifferent. Finally, the other major metabolite of ellagitannin metabolism, URO-A, displayed almost no activity in combination studies, beside its negligible effect on the MIC of penicillin G against MRSA.

Table 2: The effect of title urolithins on the MICs of reference molecules.

Reference Drug/Combination	Bacterial strain / MIC	∑FIC
	S. aureus	
Penicillin G (alone)	0.03	
Penicillin G + URO-AMM (64-512 mg/L)	0.125	> 4
	E. faecalis	
Ampicillin (alone)	0.5	
Ampicillin + URO-B (8-16 mg/L)	0.25	0.75-1
	MRSA	
Penicillin G (alone)	8	
Penicillin G + URO-B (16-128 mg/L)	32	>4
Penicillin G + URO-BM (8-32 mg/L)	16	2
Penicillin G + URO-ADM (8-16 mg/L)	16	2
Penicillin G + URO-AMM (8-512 mg/L)	16	2

CONCLUSION

There are limited number of studies conducted on the antibacterial activity of urolithins. From this perspective, this study for the first time, analyzed the antibacterial activity of major urolithins (Urolithins A and B) concomitant to their methyl ether derivatives against some Gram(+) and Gram(−) strains. In general, it was found that the antibacterial activity of urolithins was compound- and the bacterial strain-specific. Furthermore, the synergistic and antagonist activity results also depicted that some urolithins (URO-B and URO-AMM) might act as antagonist, since they were able to lower the MIC of reference drugs more than four-fold.

The study outcomes also warrant future research studies. At first hand, the activities obtained against *Enterococcus faecalis* and MRSA points out that the

urolithin scaffold is improvable to design alternative urolithin based antibacterial compounds. On the other hand, depending on the exposure level to ellagitannin-rich diet, particularly involving pomegranate juice, the urolithins, formed through metabolism and present in systemic circulation, can interfere with antibacterial drug treatment. From this perspective, the findings regarding the effects of URO-B and URO-AMM might be enlarged in future research studies to see the extrapolation of antagonist effects to other beta lactam antibiotics.

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