Egypt. J. Chem. **Vol. 64,** No. 12 pp. 7229 - 7234 (2021)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Synthesis of Novel Bivalent Hydrazino-Thymohydroquinone Analogs Derived from Thymoquinone as Potential Antimicrobial Agents

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Abstract

In our previous work, it was reported that thymoquinone (TQ) and some thymohydroquinone (THQ) derivatives were used as precursors for the synthesis of potential anticancer agents. Hence, as a part of our ongoing program in the design of biologically active compounds derived from TQ scaffold we herein investigated the synthesis of homo- and heteronuclear thymol dimers tethered through various linkers such as ethylene, butylene, acetyl and N,N'-acetyl piperazine groups. The newly prepared compounds were examined as antimicrobial agents. Compounds **7** and **8** bearing a piperazine moiety exhibited highest antibacterial activity comparing to TQ, THQ and ampicillin especially against Staphylococcus aureus bacteria with MIC value of 16 µg/mL. All compounds showed moderate antifungal activity against Candida albicans and Aspergillus fumigatus. Together, the new TQ-based bivalent lead might be serving as a promising scaffold for development of novel and potent antimicrobial agents.

Keywords: Thymohydroquinone; Antimicrobial; Bivalent, Heteronuclear dimer

1. Introduction

Thymoquinone (TQ, **1**) is a natural product existed in the seeds of Nigella sativa L. has been recognized as a key bioactive component in various medical purposes [1]. Several studies reported the derivatization of TQ through adding substitutions to TQ ring or by the reduction of quinones to quinol forms can leads to enhance the therapeutic effect of TQ such as treatment of cancer, malaria, microbial growth, and others [2-10]. This broad array of usage has led to the increased interest in its various derivatives. Of interest is the hydrazino-thymohydroquinone (HTHQ) derivative which was developed at the first time by our research group (**Figure 1**) [2].

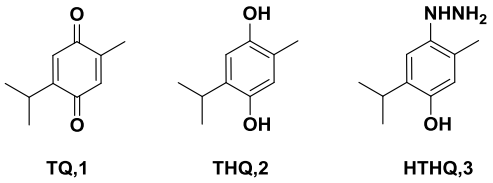

Figure 1. Structure of thymoquinone and its derivatives

This is partly due to the presence of the hydrazine moiety in this compound, for which open the access to design biologically active nitrogen compounds

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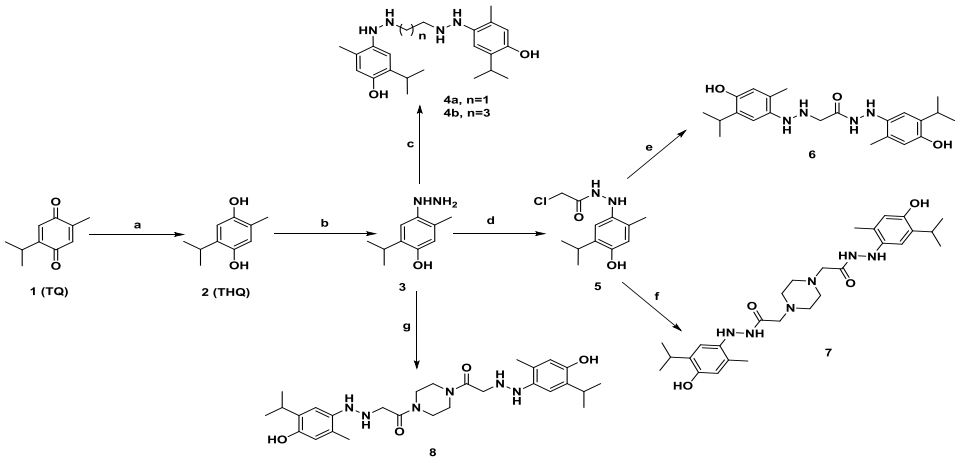
Receive Date: 17 May 2021, Revise Date: 19 June 2021, Accept Date: 28 June 2021
DOI: 10.21608/EJCHEM.2021.74154.3743

based on phenolic unit. In continuation to our previous work, we aimed to exploit the hydrazine group in HTHQ (**3**) to synthesize new bivalent compounds to be employed as potential therapeutic agents [11-13]. As a result of resistance to current drugs and emerging new diseases there is constant need of obtaining antimicrobial and anticancer agents with minimal side effects. Aiming to improve the bioavailability of TQ and THQ, herein in this protocol we aimed to design potent antimicrobial agents derived from TQ. Six THQ analogs including nitrogen-substituted derivatives were synthesized with different substituted groups (e.g. variation of length of alkyl chains and the use of piperazine as linker) were produced. Both the synthetic and the as-prepared TQ analogs were analyzed as antimicrobial agents

2. Results and discussions

2.1. Chemistry

A series of reduced thymoquinone analogous TQ (**4a,b**, **5**, **6**, **7** and **8**) were synthesized with different substituted groups (e.g. variation of length of alkyl chains and incorporation of piperazine ring as linker), Scheme 1.



Scheme 1. Synthesis of target reduced thymoquinone derivatives. Reagents and reaction conditions: a) $NaBH_4$, reflux, EtOH; b) NH_2NH_2, EatOH, c) $Br(CH_2)_nBr$; n=2 or 4, THF, 60 ^oC, 5h, d) chloroacetylchloride, EtOH, 25 ^oC, 2h, e) hydrazine-TQ **3**, f) piperazine g) dichloroacetyl piprazine THF, 60 ^oC, 5h.

Reduced thymoquinone (THQ, **2**) was obtained by reduction of thymoquinone with excess of sodium boronhydride, whereas hydrazino-thymoquinone (HTHQ, **3**) was yielded by reacting TQ (**1**) or THQ (2) with excess hydrazine hydrate in ethanol [2]. HTQH (**3**) was used in this protocol as intermediated compound for the synthesis of some hetero- or homo-bivalent products containing reduced thymo-hydroquinone moiety. Compound **4a** and **4b** were successfully synthesized in excellent yields (86% and 92% isolated yields, respectively through the reaction of HQTH with dibromoethane and dibromobutane, respectively. As a comparison, two ^1H NMR spectra of (HTHQ, 3) and 4,4'-(ethane-1,2-diylbis(hydrazine-2,1-diyl))bis(2-isopropyl-5-methyl-phenol) (**4a**) are presented in a Figure 2. It was observed that the appearance of multiplet peak at 3.61-3.44 ppm confirmed the presence of two CH_2 groups linked between two HTHQ molecules.

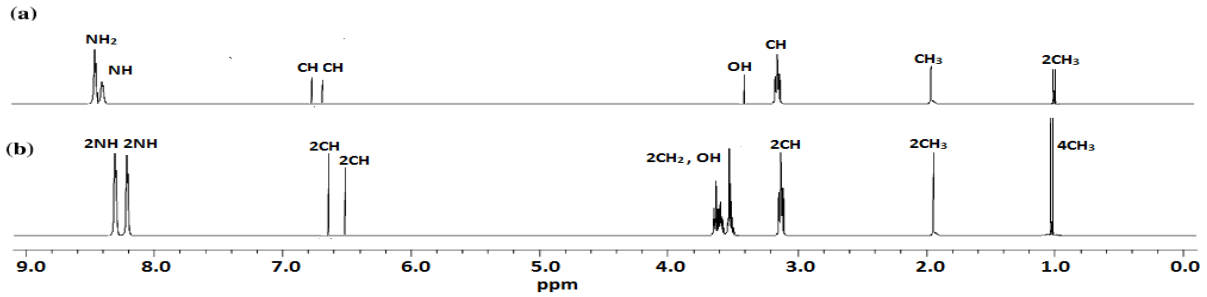


Figure 2. ^1H NMR of (a) compound **3**; (b) compound **4a**.

Moreover, compound 3 was reacted with chloroacetyl chloride affording compound **5** with yield 84%. This compound was reacted with another molecule of HTQH to produce product **6** in 82% isolated yield. Also, by reacting compound **5** with piperazine, product **7** was obtained in good yield (87%). Finally, compound **3** was reacted with bis-chloroacetyl piperazine to afford compound **8** in 85% isolated yield. The ^{1}H NMR and ^{13}C NMR spectroscopic data were in accordance with the assigned structures.

2.2. Biological Screening

2.2.1. Antimicrobial Evaluation

The as-synthesized analogs were investigated for their antimicrobial potential against a panel of bacteria and fungi species including Staphylococcus aureus (ATCC-29213), Escherichia coli (ATCC-25922), Klebsiella pneumoniae (ATCC-700603), Pseudomonas aeruginosa (ATCC 27853), Candida albicans (ATCC 10231), Aspergillus fumigatus (clinical isolate). The antibacterial and antifungal results were expressed in terms of the diameter of inhibition zones (Tables 1) and the minimum inhibitory concentration (MIC, µg/mL) was measured as well using ampicillin and fluconazole as reference drugs, Tables 2. In general, the tested TQ-based derivatives exhibited remarkable antibacterial activities compared to their antifungal potential activity.

2.2.2. Antibacterial activity

From the examination of antibacterial assay results, it was found that almost all the tested compounds exhibited strong or moderate activity against the bacteria strains used in the screening assay except compounds **4a,b** with the alkyl linker showed the least activity. Generally, the new compounds revealed higher activity against Gram-positive stains (S. aureus) more than Gram-negative bacteria used in the test. Initially, the chemical modification of THQ, **2** by incorporating a hydrazino moiety as in compound **3** resulted in maintaining the activity comparing with TQ, **1** with slightly enhancement in the activity against Staphylococcus aureus, Klebsiella pneumoniae and Pseudomonas aeruginosa over THQ, **2**. It was noticed an improvement in the activity upon changing the alkyl linker into acetyl one as in compound **6**. However, there was a remarkable increase in the antibacterial activity upon incorporating a piperazine moiety as a part of the linker in compounds **7** and **8**. Both compounds were the most potent derivatives in the series against all tested bacteria including Staphylococcus aureus in particular. It was noteworthy that the antibacterial activity of these two compounds 7 and 8 against Staphylococcus aureus (MIC = 16 µg/mL) was better than TQ, THQ and ampicillin with MIC values of 64, 128, 32 µg/mL, respectively, Table 2. On the contrary, the newly synthesized compounds exhibited moderate to weak activity as antifungal agents comparing with fluconazole as a reference drug. It worth mentioned that any chemical modification in THQ **2** structure influenced negatively the potency in general. Examining the results revealed that compounds 3 and 8 were the most active derivatives against Candida albicans with MIC value of 128 µg/mL. The rest of compounds showed weak to moderate activities against both used fungi stains (Candida albicans and Aspergillus fumigatus) with MIC values between 256-512 µg/mL, Table 2.

Table 1: Inhibition zone values of the newly synthesized thymoquinone derivatives 1-8 compared with ampicillin and Fluconazole as reference drugs.

Compound code	Inhibition Zone (mm)					
	Antibacterial Activity				Antifungal Activity	
	Sa	***Ec***	***Kp***	***Pa***	***Ca***	***Af***
1 (TQ)	+++	+++	++	+	++	++
2 (THQ)	++	+++	++	++	+	++
3	+++	++	++	++	++	+
4a	+	++	+	+	+	+
4b	+	++	+	-	++	+
6	+++	+++	+	++	+	++
7	++++	++++	+++	+++	++	+
8	++++	++++	++++	++	+	++
Ampicillin	++++	+++	+++	++	NT	NT
Fluconazole	NT	NT	NT	NT	++++	++++

Results are interpreted in terms of the diameter of the inhibition zone: (-) inactive, (+) < 10 mm, (++) 10-15 mm, (+++) > 16-20 mm, (++++) > 20, NT: Not Tested. Sa: Staphylococcus aureus (ATCC-29213), Ec: Escherichia coli (ATCC-25922), Kp: Klebsiella pneumoniae (ATCC-700603), Pa: Pseudomonas aeruginosa (ATCC-27853), Ca: Candida albicans (ATCC-10231), Af: Aspergillus fumigatus clinical isolate.

Table 2: MIC values of the newly synthesized thymoquinone derivatives 1-8 compared with ampicillin and Fluconazole as reference drugs.

Compound code	Minimum Inhibitory Concentration (MIC) µg/mL					
	Antibacterial activity				Antifungal activity	
	Sa	***Ec***	***Kp***	***Pa***	***Ca***	***Af***
1	64	64	128	256	256	256
2	128	64	512	512	128	128
3	64	64	128	128	128	256
4a	512	256	512	512	256	512
4b	512	512	512	-	512	256
6	128	64	128	256	256	256
7	16	64	64	128	256	256
8	16	64	128	128	128	256
Ampicillin	32	32	64	128	NT	NT
Fluconazole	NT	NT	NT	NT	4	8

MIC: minimum inhibitory concentration; NT: Not Tested; (-): denotes inactive. Sa: Staphylococcus aureus ATCC 29213, Ec: Escherichia coli ATCC 25922, Kp: Klebsiella pneumoniae ATCC-700603, Pa: Pseudomonas aeruginosa ATCC 27853, Ca: Candida albicans ATCC 10231, Af: Aspergillus fumigatus clinical isolate.

3. Experimental Protocols

3.1. Chemistry

Chemical reagents and solvents were purchased from Sigma-Aldrich Company and used without purification. ^{1}H NMR and ^{13}C NMR spectra were measured on Bruker APX400 spectrometer at 400 MHz and 101 MHz respectively in DMSO-d_6. Chemical shifts were reported on the δ scale and J values were given in ppm and Hz; respectively. Thin layer chromatography (TLC) was done by silica gel plates 60 GF254, cellulose plates (20×20 cm) from Sigma-Aldrich. High-resolution mass spectrometry (HRMS) analysis was measured using a 6230 Series Accurate-Mass Time-Of-Flight (TOF) liquid chromatography (LC)/MS system. MacConkey's agar, Muller-Hinton agar (MHA), Muller-Hinton broth, sabouraud dextrose agar (SDA), SDA broth and nutrient agar were supplied from Oxoid, Ltd, England. Sterile Petri dishes were supplied from Nuova Aptaca SRL, Italy. Sterile 96 well plates were supplied from Thermo scientific, U.S.A. multichannel digital micropipette and pipettes of different volumes were supplied from Eppendorf, U.S.A. White tips (200µl) were supplied from Bipointe, U.S.A.

3.1.1. General procedure (A) for the synthesis of compounds 4a, 4b and 8.

Mixture of 4-hydrazinothymohydroquinone (**3**) (2.2 mmol), dibromoethane (1 mmol), dibromobutane or dichloroacetyl piprazine (1 mmol), in THF (30 mL) was heated at 60 oC for 5 h. After completion of the reaction, the reaction mixture was left to cool at room temperature followed by the addition of water (30 mL) and then extracted with EtOAc (3x25 mL). The collected organic layers were dried (Na2SO4). The solvent was evaporated using rotavap. The obtained residue was purified by short plug column chromatography using SiO_2 and (ethyl acetate/hexane, 10%, 20%, 30%, 40%) as eluent.

3.1.2. General procedure (B) for the synthesis of compounds 6 and 7.

4. Mixture of 4-hydrazinothymohydroquinone (3) (1 mmol), chloroacetyl chloride (1 mmol), in EtOH (30 mL) was stirred at room temperature for 2 h. The formed precipitated product was collected by filtration and it was used without further purification for the next step. ^{1}H NMR of compound 5 (400 MHz, DMSO-d_6): δ (ppm) 9.65 (br, s, 1H, NH), 8.29 (br, s, 1H, NH), 6.73 (s, 1H), 6.64 (s, 1H), 4.49 (s, CH_2), 3.16 (hept, J= 6.8 Hz, 2H, CH), 1.97 (s, 3H, CH_3), 1.03 (d, J = 6.8 Hz, 6H, $2CH_3$). After completion of the reaction, the reaction mixture was left to cool at room temperature followed by the addition of water (30 mL) and then extracted with EtOAc (3x25 mL). The collected organic layers were dried (Na_2SO_4). The solvent was evaporated using rotavap. The obtained residue was purified by short plug column chromatography using SiO_2 and (ethyl acetate/hexane, 10%, 20%, 30%, 40%) as eluent.

4,4'-(Ethane-1,2-diylbis(hydrazine-2,1-diyl))bis(2-isopropyl-5-methylphenol), (4a). The title compound was prepared using the general procedure A. Yield 86%. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 8.34 (s, 2H, 2NH), 8.21 (br, s, 2H, 2NH) 6.72 (s, 1H), 6.55 (s, 1H), 3.61-3.44 (m, 5H, $2CH_2$, OH), 3.13 (hept, J= 8.6 Hz, 2H, CH), 1.96 (s, 6H, CH_3) 1.03 (d, J = 6.8 Hz, 12H, $4CH_3$); ^{13}C NMR (101 MHZ, DMSO-d_6): δ (ppm) 149.3 (2C), 146.2 (2C), 133.4 (2C), 121.8 (2C), 117.6 (2CH), 113.5 (2CH), 49.2 ($2CH_2$), 26.7 (2CH), 24.3 ($4CH_3$), 15.2 ($2CH_3$).

m/z (HRMS): calcd for $C_{22}H_{34}N_4O_2$: 386.2682, found: 387.2906 $[M+H]^+$.

4,4'-(Butane-1,4-diylbis(hydrazine-2,1-diyl))bis(2-isopropyl-5-methylphenol), (4b). The title compound was prepared using the general procedure A. Yield 83%. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 8.32 (s, 2H, 2NH), 8.15 (br, s, 2H, 2NH), 6.79 (s, 1H), 6.64 (s, 1H), 3.67 (t, J= 6.8 Hz, 2H, CH_2), 3.57-2.40 (m, 5H, $2CH_2$, OH), 3.05 (hept, J= 6.8 Hz, 2H, CH), 2.01 (s, 3H, CH3), 1.10 (d, J = 6.8 Hz, 6H, 2CH3); ^{13}C NMR (101 MHZ, DMSO-d_6): δ (ppm) 148.7 (2C), 146.8 (2C), 133.2 (2C), 122.7 (2C), 116.3 (2CH), 112.9 (2CH), 50.1 ($2CH_2$), 26.5 (CH), 23.8 ($4CH_3$), 14.8 ($2CH_3$). m/z (HRMS): calcd for $C_{24}H_{38}N_4O_2$: 414.2995, found: 415.2598 $[M+H]^+$.

N'-(4-hydroxy-5-isopropyl-2-methylphenyl)-2-(2-(4-hydroxy-5-isopropyl-2-methylphenyl)-hydrazineyl)acetohydrazide, (6). The title compound was prepared using the general procedure A. Yield 89%. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 9.76 (br, s, 2H, NH), 8.33 (br, s, 2H, 2NH), 8.25 (br, s, 1H, NH), 6.75 (s, 1H), 6.62 (s, 1H), 4.51 (s, CH_2), 3.18 (hept, J= 6.8 Hz, 2H, CH), 2.03 (s, 3H, CH3), 1.05 (d, J = 6.8 Hz, 6H, $2CH_3$); ^{13}C NMR (101 MHZ, DMSO- d_6) δ (ppm) 166.8 (C=O), 148.7 (2C), 146.1 (2C), 133.1 (2C), 121.8 (2C), 116.4 (2CH), 112.9 (2CH), 40.6 (CH_2), 25.8 (2CH), 24.3 ($4CH_3$), 15.6 ($2CH_3$). m/z (HRMS): calcd for $C_{22}H_{32}N_4O_3$: 400.2474, found: 401.2402 $[M+H]^+$.

2,2'-(piperazine-1,4-diyl)bis(N'-(4-hydroxy-5-isopropyl-2-methylphenyl)acetohydrazide), (7). The title compound was prepared using the general procedure A. Yield 87%. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 9.68 (s ,2H, 2NH), 8.37 (br, s, 2H, 2NH), 6.72 (d, J = Hz, 1H), 6.50 (d, J = Hz, 1H), 4.63 (s, 2H, CH_2), 2.51 (s, 8H, $4CH_2$), 2.42 (br, s, 1H, OH), 3.11 (hept, J = 6.8 Hz, 2H, CH), 1.97 (s, 3H, CH_3), 1.09 (d, J = 6.8 Hz, 6H, 2CH3); ^{13}C NMR (101 MHZ, DMSO-d_6) δ (ppm) 166.5 (C=O), 149.2 (2C), 147.0 (2C), 132.6 (2C), 122.7 (2C), 117.1 (2CH), 112.9 (2CH), 42.1 ($2CH_2$), 46.8 (4CH2), 26.1 (2CH), 23.8 (4CH3), 15.8 ($2CH_3$). m/z (HRMS): calcd for $C_{28}H_{42}N_6O_4$: 526.3268, found: 527.2975 $[M+H]^+$.

1,1'-(piperazine-1,4-diyl)bis(2-(2-(4-hydroxy-5-isopropyl-2-methylphenyl)hydrazineyl)ethan-1-one), (8). The title compound was prepared using the general procedure A. Yield 91%. ^{1}H NMR (400 MHz, DMSO-d_6), δ (ppm) 8.70 (s, 1H, NH), 8.32 (s, br, 2H, 2NH), 8.20 (br, s, 1H, NH), 6.74 (s, 1H), 6.65 (s, 1H), 4.23 (s, 2H, CH_2), 3.41 (br, s, 1H, OH), 2.48 (s, 8H, $4CH_2$), 3.10 (hept, J= 6.8 Hz, 2H, CH), 1.98 (s, 3H, CH_3), 1.07 (d, J = 6.8 Hz, 6H, $2CH_3$); ^{13}C NMR (101 MHz, DMSO- d_6): δ (ppm) 167.2 (C=O), 148.6 (2C), 147.0 (2C), 132.7 (2C), 122.1 (2C), 118.2 (2CH), 113.5 (2CH), 51.3 (2C), 46.5 ($4CH_2$), 25.6 (2CH), 24.7 ($4CH_3$), 15.2 ($2CH_3$). m/z (HRMS): calcd for $C_{28}H_{42}N_6O_4$: 526.3268, found: 527.3031 $[M+H]^+$.

Antimicrobial evaluation

Antimicrobial susceptibility testing (AST) was determined by Kirby-Bauer diffusion [15]. Broth micro-dilution method was also performed using cation modified Mueller-Hinton broth to determine the MIC values of tested compounds by Kirby-Bauer disc diffusion [16].

4. Conclusions

The design and synthesis of improved antimicrobial agents continues based on thymoquinone and its derivatives to be an important line in the discovery of new therapeutic agents derived from natural sources. In this context the newly synthesized reduced thymoquinones have analyzed for their potential antimicrobial effects. It was determined that the dimer compounds 7 and 8 contained piprazine ring as spacer were exhibited higher antibacterial agents compared to ampicilline as reference drug. However, the all compounds showed moderate antifungal activity. These results shed light on the importance of the discovery of new antibacterial and antifungal agents based on bivalent compounds that derived from thymoquinone as natural product.

5. Acknowledgment

The current work was supported by Taif University Researchers Supporting Project number (TURSP-2020/29), Taif University, Taif, Saudi Arabia.

6. Authors' contributions

YM and AA carried out the chemistry work, participated in the sequence, alignment and drafted manuscript. ME and HA carried out the Biology work and revision of the manuscript.

7. Conflicts of interest

"There are no conflicts to declare".

8. References

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