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Synthesis and Investigation of Mutagenic and Genotoxic Effects of Some 2-hydroxy-1,4-Naphthoquinone Derivatives by Ames and Comet Assay

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Authors' contributions

This work was carried out in collaboration between all authors. Authors RL, IHC and OA designed the study and prepared the manuscript. Author RL managed the literature searches. Author OA prepared 2-hydroxy-1,4-naphthoquinone derivatives*. All authors read and approved the final manuscript.*

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ABSTRACT

In this study, mutagenic and genotoxic effects of novel 2-hydroxy-1,4-naphthoquinone (1) derivatives, 2-phenyl-2-(2-thiophenyl)-2,3-dihydronaphtho[2,3-b]furan-4,9-dione (3) and 2-hydroxy-3-[(E and Z)-2-phenyl-2-(2-thiophenyl)ethenyl]naphthalene-1,4-dione (4) were investigated by using bacterial reverse mutation assay in *Salmonella typhimurium* TA98 and TA100 strains with or without metabolic activation system (S9 mix) and comet assay in haploid *Saccharomyces*

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cerevisiae BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*), respectively. Derivatives, 3 and 4 were dissolved in dimethyl sulfoxide (DMSO) for all test systems. Five non-cytotoxic concentrations of the derivatives were tested in two parallel independent experiments in Ames test. Ames test did not show mutagenicity of test compounds. Two different concentrations (50 µg/m L and 100 µg/mL) of 3 and 4 were applied to *S. cerevisiae* cells. It was found that test materials did not show genotoxic effect. While all of the 4 and 100 mM of concentration 3 showed protective effect, all of the 1 and 50 mM of 3 did not show a protective effect against the DNA damage generated by H_2O_2.

Keywords: Naphthoquinone; ames; comet; yeast; genotoxicity.

1. INTRODUCTION

Henna (*Lawsonia alba* or *Lawsonia inermis*, contains 1.0–1.4% 2-hydroxy-1,4-naphthoquinone) is a flowering plant, which has been used to dye hair, skin, fingernails, leather, silk and wool from more than 5000 years [1-3]. Some 1,4-naphthoquinone derivatives are biologically active molecules because of its antifungal, antibacterial, anticancer, antiproliferative, antiplatelet, antiinflammatory, antileishmania, antiallergic, antimalarial and antiviral activities [4-15]. This biological activity corresponds by gaining the one and/or two electrons to make the related dianion species or radical anion. These 1,4-naphthoquinones generate the formation of reactive oxygen species (ROS), including hydrogen peroxide and superoxide anions, which damage the DNA and certain essential proteins [15,16]. Due to it's widespread use and pharmacological applications, trend of its new synthetic derivatives has been progressed.

The bacterial reverse mutation assay, also known as Ames test and *Salmonella*/microsome test, is more reliable, rapid and cheap short-term bacterial test systems, and widely used for examining the mutagenicity of chemical(s) to detect various types of gene mutations [17-20].

The yeast comet assay, a sensitive, fast and inexpensive test, is used to determine oxidative DNA damage, genotoxic or protective effects of chemicals and DNA damage repair in the eukaryotic cells [21-25].

The objective of this study was to investigate the mutagenic and genotoxic effects of **3** and **4** by employing both in *S. typhimurium* TA98 and TA100 strains with or without S9 mix and in haploid *S. cerevisiae* BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*), respectively.

2. MATERIALS AND METHODS

2.1 Organisms

The LT-2 TA98 and TA100 histidine-demanding auxotrophs of *S. typhimurium* were received from Prof. N.Diril, Hacettepe University, Turkey.

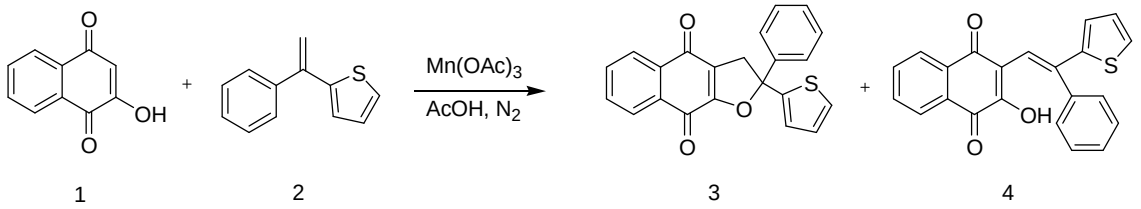
The yeast strain, haploid *S. cerevisiae* BY4741 (*MATahis3* D1 leu2D0 met15 D0 ura3D0) was provided by Prof. Rui Pedro Soares Oliveira, Biology Department of Minho University, Portuguese.

2.2 Chemicals

2-hydroxy-1,4-naphthoquinone, S9 from Liver from rat (Sprague-Dawley), bacto agar, nutrient broth no:2 oxoid and 2-aminoanthracene (2AA, CAS No. 613-13-8) were purchased from Sigma Aldrich. 4-nitro-o-phenylendiamine (NPD, CAS No. 99-56-9), 2-aminofluorene (2AF, CAS No. 153-78-6), L-histidine HCl, D-biotin, ampicillin trihydrate, D-glucose 6-phosphate and β-nicotinamide adenine dinucleotide phosphate were bought from Fluka. Citric acid monohydrate, sodium hydroxide, sodium azide (SA, CAS No. 26628-22-8), potassium chloride, sodium chloride and DMSO were purchased from Riedel. **2** was prepared by dehydration reaction from the carbinole formed by Grignard reaction of arylmagnesium bromide and suitable carbonyl compounds [26]. All other chemicals used for both assays were of analytical quality and bought from Sigma–Aldrich Company.

2.3 Experimental Procedure

A solution of 2.5 mmol $Mn(OAc)_3$ (0.67 g) in glacial AcOH (10 mL) was mixed and heated (80℃) under N_2, till dissolution. Then the solution was allowed to cool till 65℃. The 1 mmol



1 2 3 4

Fig. 1. Synhtesis of novel 2-hydroxy-1,4-naphthoquinone (1) derivatives, 2: aklene, 3: 2-phenyl-2-(2-thiophenyl)-2,3-dihydronaphtho[2,3-b]furan-4,9-dione, 4: 2-hydroxy-3-[(E and Z)-2-phenyl-2-(2-thiophenyl)ethenyl]naphthalene-1,4-dione

solution of 2-hydroxy-1,4-naphthoquinone (**1**) and 1.25 mmol alkene (**2**) were added in 5 mL AcOH. The reaction was completed in 10 minutes or 24 hours. After that, 20 mL distilled water was poured, and extraction of mixture was made by $CHCl_3$ (3x20 mL). Neutralization of organic phases was done with saturated $NaHCO_3$ and dried (anhydrous Na_2SO_4), finally completed this phase by evaporation. Purification of crude products (**3** and **4**) was carried out by column chromatography with hexane–EtOAc5:1 as a flint (Fig. 1). Yield for compound **3** (reaction time was 10 min.) was 75%, whereas, yield for compound **4** (E and Z isomer mixing; reaction time was 24 hours) was 60%.

2.4 Ames Plate Incorporation Test

Preparation of the stock *S. typhimurium*TA98 (his D3052, *rfa*, Δ*uvrB*, +R), TA100 (his G46, *rfa*, Δ*uvrB*, +R) strains and phenotypic properties of these strains, including histidine requirement, presence of *R*-factor, *rfa* mutation*, uvr*B mutation and number of spontaneous revertants were checked, as described by Maron and Ames [27] and preserved at -80℃. The concentrations of test solutions (10000, 1000, 100, 10, 1 and 0.1 µg/plate) were based on a preliminary toxicity test according to the Dean et al. [28].

Mutagenicity was investigated by using a plate incorporation assay of the Ames test with *S. typhimurium* strains TA98 to find frame shift mutations and TA100 to investigate base pair exchanges with or without S9 mix in accordance with the procedure described by Maron and Ames [27]. Strains selection and their testing were done by the method of Mortelmans and Zeiger [29]. As a positive controls, NPD (200 µg/plate) for TA98 and SA (10 µg/plate) for TA100 without S9 mix, 2AF (200 µg/plate) for TA98 and 2AA (5 µg/plate) for TA100 with S9 mix were used as standard mutagens.

Briefly, a sterile tube of 2.0 ml top agar (kept 45℃), 100 µL of test substance, 100 µL of a cell suspension from an overnight culture (1-2x10^9 cells/mL) and 500 µl of S9 mix (or 500 µL phosphate buffer). Following vortexing for 3 seconds, the mixture was added into minimal glucose agar plates and incubated for 72 h at 37℃. Then, counting of revertant colonies was done. Concentrations of 100, 50, 25, 12.5 and 6.25 µg/plate for TA98 and TA100 with or without S9 mix were used. Experiments were performed in triplicate in two independent parallel experiments.

The results of the Ames test for genotoxicity testing of chemicals were interpreted as described by the United States Environmental Protection Agency [30]. A test chemical was declared as mutagenic, where concentration dependent relationship or two or higher number of revertant colonies over the solvent control were observed in strains [29].

2.5 Alkaline Comet Assay

Stock cultures of this yeast strain were grown and preserved in YPD medium (2% peptone, 1% yeast extract, 2% agar and 2% glucose) at 27℃. The DNA damage level was detected in *S. cerevisiae* strain BY4741 by alkaline comet assay. Yeast cells were taken, maintained in 10 ml of YPD medium and incubated for 24 hours at 30℃, 200 rpm. Suitable volume of pre-inoculum was taken and dilution was made to get 25 ml culture with an absorbance value of 600 0.1. Again, it was incubated overnight under 30℃, 200 rpm until to get absorbance of 600 0.4–0.8. Cells were centrifuging for 2 min at 4℃, 5000 rpm, and washed twice with the cold deionized H_2O. Suspension of pellet was made with the equal volume of Sorbitol buffer (25 mM KH_2PO_4, 1 M sorbitol, pH 6.5) at 4^oC. Cell suspensions (1 mL) were obtained, after centrifugation for 2 min at 15300 rpm, 4℃. Lyticase buffer was added in cells (2 mg/mL lyticase, 300 µL deionized H_2O, 50 mM β-mercaptoethanol, 500 µL S buffer 2x), incubated for 30 min at 200 rpm 30℃ to get

spheroplasts. Cells suspension (80 µl) was spread by the each aliquot. Spheroplasts were obtained by centrifugation for 2 min at 15300 rpm (4℃). The cells were mixed by adding 80 µL Low melting agarose (1.5%) (w/v in S buffer) at 35℃. The cell mixture was layered onto normal melting agarose (0.5%) coated slides and covered with coverslips. After that, 300 µL oxidant solution (10 mM H_2O_2) was spread on each slide after removing the coverslips. The slides were kept at 4℃ for 20 min and washed with S buffer for 4-5 min. The slides were kept for 20 min in a cold lysing solution (30 mM NaOH, 1 M NaCl, 0.05% (w/v) laurylsarcosine, 10 mM Tris–HCl, 50 mM EDTA; pH 10). Then, slides were washed with buffer (10 mM EDTA, 30 mM NaOH, 10 mMTris–HCl and pH kept as > 12.3) for 20 min. Electrophoresis was performed with electrophoresis buffer solution at at 0.7 V/cm for 10 min. The neutralization of gel was done by deionized H_2O. Lastly, slides were stained with 60 µL ethidium bromide (2 µg/mL) and analyzed under fluorescence microscope. Numbers of comets were scored from 0-4 according to the extent of damage.

2.6 Statistical Analysis

The results were presented as mean ± standard deviation for all tested concentrations. The test results were performed with SPSS 18.0 version for Windows software. The Ames test was analyzed by Mann-Whitney test. The Duncan multiple range test was used for yeast comet test. The differences were considered significant at $p \leq 0.05$.

3. RESULTS

3.1 Ames Plate Incorporation Test

The Ames test has been developed in the past to screen compounds for their capacity to induce frame shift mutation and/or base pair mutations using specific *S. typhimurium* (his^-) strains. The results from the Ames test are shown in Table 1. Non-cytotoxic concentrations of sample solutions were found by the protocol of Dean et al. [28]. According to the results obtained, cytotoxicity was observed at concentrations higher than 100 µg/plate of test solutions. Therefore, non-cytotoxic concentrations less than this were taken.

The average revertant colony numbers in solvent control were 23.4±2.96 for TA98 and 96.8±2.94 for TA100 in the absence of S9 mix and 23.4±0.89 and 97.6±1.94 in the presence of S9 mix, respectively. Spontaneous revertants values were within the normal range for the both strains. Slightly lower values were observed in some concentrations, compared to solvent control spontaneous revertants. But positive control mutagens (SA, NPD, 2AF, and 2AA) showed increase values than the spontaneous mutation. Highest value (158.2±6.72) was observed in the TA100 with S9 mix at 100 µg/plate concentration of 1, and lowest (14.2±1.09) was there in the TA98 with S9 mix of at 100 µg/plate concentration of **3**. The results were significant, $p<0.05$ (Mann-Whitney test) in the TA98 without S9 mix and in the TA100 with and without S9 mix relative to solvent control group. Revertant colony numbers were decreased by the application of S9 mix in TA98. In contrary to this, revertant colony numbers were increased in S9 mix in TA100 except for 100 and 50 µg/plate concentration of **3**, 12.5 and 6.25 µg/plate concentration of **4**. After applying of 5 different concentrations of the test solutions, there were no induced revertants along the concentration range tested in either with or without S9 mix in both tested strains.

3.2 Yeast Comet Assay

The genotoxic potential of yeast cells at different concentrations is given in Table 2. The highest DNA damage score was observed by the positive control H_2O_2 at 10 mM. The increase in DNA damage score in the positive control group compared to the negative control group was statistically significant. The DNA damage scores in test chemical **1** were 44.33±6.5 and 48±4 for 50 mM and 100 mM, respectively. This increase in DNA damage in substance **1** was not statistically significant as compared to negative control group. Similarly, **3** and **4** chemicals induced DNA damage, but this increase was not statistically significant. It can be said that these materials did not show genotoxic activity.

Protective potential against H_2O_2-induced DNA damage in yeast cells by different concentrations of test substances are given in Table 3. The test chemical **1** failed to show effective protection effect against DNA damage. There was no significant difference ($p \leq 0.05$) between positive control group and the test chemical $1+H_2O_2$ group. The protective potential of chemical **3** by the concentrations of 50 and 100 mM in the form of DNA damage score was found 81±1.73 and 73.66±3.21, respectively. Test chemical 4 as

Table 1. Mutagenicity of 2-hydroxy-1,4-naphthoquinone derivatives in *S. typhimurium* TA98 and TA100 strain with or without S9 mix

Agent	Amount (µg/plate)	No of his$^+$ revertants/plate Mean ± SD*			
		TA98		TA100	
		- S9	+ S9	- S9	+ S9
1	100	22.8±2.28	20.2±2.16*	113.2±5.31*	158.2±6.72*
	50	22.6±1.51	18±1.22*	119±2.44*	122.4±5.63*
	25	24.6±2.7	20±083*	122.2±6.34*	136.2±7.75*
	12.5	23.8±2.38	18±0.7*	120.8±1.64*	125.4±3.97*
	6.25	25±2.64	17.8±1.09*	118.8±4.76*	133.8±6.64*
3	100	21.6±2.07	14.2±1.09*	136.2±8.7*	113.2±5.97*
	50	18.2±1.3*	18.2±0.83*	137±5.56*	133.8±5.44*
	25	22.4±1.67	16.6±0.89*	146.6±6.58*	158±6.04*
	12.5	23.6±2.07	14.6±1.14*	126.4±4.82*	130.2±9.06*
	6.25	25.8±2.28	15.8±1.3*	135.4±4.31*	139±10.34*
4	100	20.8±1.64	17.2±2.16*	124.8±4.43*	131.8±9.88*
	50	24±1.87	17.2±1.09*	123.4±4.97*	129±6.24*
	25	23.6±2.4	18.6±1.14*	122.8±8.34*	128.8±3.19*
	12.5	21.2±2.16	15.6±1.67*	114±2.73*	110.6±5.77*
	6.25	22.8±2.68	19±0.7*	97.6±5.50*	102±4.69*
Solvent control		23.4±2.96	23.4±0.89	96.8±2.94	97.6±1.94
SA	10			1475.6±241.02*	
2AA	5				2201.4±170.87*
2AF	200		511±59.13*		
NPD	200	1916.8±144.06*			

** Statistically significant at p< 0.05 (Mann-Whitney test), SD: Standard deviation, **1**: 2-hydroxy-1,4-naphthoquinone, **3**: 2-phenyl-2-(2-thiophenyl)-2,3-dihydronaphtho[2,3-b]furan-4,9-dione, **4**: 2-hydroxy-3-[(E and Z)-2-phenyl-2-(2-thiophenyl)ethenyl]naphthalene-1,4-dione, SA: Sodium azide, 2AA: 2-aminoanthracene, 2AF: 2-aminofluorene, NPD: 4-nitro-o-phenylenediamine*

compared to other test chemicals. Test chemical **3** and **4** along with H_2O_2 showed significant difference as compared to the positive control group. Moreover, it was observed that by increasing the concentration of chemical (100 mM) the protective effect was also increased showed more protective effects on DNA damage.

4. DISCUSSION

Manganese (III) acetate mediated free radical reaction, is one of the best well-known synthetic method for the synthesis of biologically active and important organic molecules through oxidation, cyclization reactions and addition which is generally difficult to attain through the established synthetic operations due to their specific, selective and mild reaction facilities [26,31]. Therefore, compounds **3** and **4** were synthesized with one-pot reaction using Mn(III) acetate.

Bacterial mutagenicity was assessed in *S. typhimurium* tester strain TA98 for detection of frame shift mutation and the test strain TA100 for measurement of base-pair substitution. The result of the Ames test showed that all tested materials were not mutagenic *S. typhimurium* TA98 and TA100 with and without S9 mix. Some concentrations of test solutions lowered the spontaneous reversion slightly as compared to the positive control mutagens (SA, 2AF, NPD and 2AA) which showed significant increases relative to the spontaneous mutation rate in the two tested strains. Revertant colony numbers in TA98 became lower when S9 mix was added. No dose-response relationship was found among five different concentrations in either with or without S9 mix with two strains. These results are in agreement with other studies performed with 1 in Ames test [32-34]. Kitagawa et al. [35] also showed that naphthoquinone didn't cause mutations by using the Ames test. However, previous literature reported the mutagenicity of some quinones and naphthoquinones after metabolization. Tikkanen et al. [36] concluded mutagenicity of naphthoquinones having 1 or 2 hydroxyl and/or methyl substituents along with

metabolic activation. It determined that the mutagenicity of quinones seems to be due to reduction of one-electron of quinones to semiquinones through hydrogen peroxide (H_2O_2) and superoxide formation [37]. These findings conclude that, number and position of substituents play key role in determining the mutagenicity of chemicals [35]. It seems that the absence of mutagenicity in our synthetic compounds dihydronaphthoquinones is due to absence of methyl and hydroxyl substituents.

Table 2. Detection of DNA damage in yeast cells, exposure to 2-hydroxy-1,4-naphthoquinone derivatives by using the comet assay

Test chemicals	Amount (mM)	DNA damage Abritrary Unit± SD
Negative control	-	35,66±4,04a
H_2O_2	10	83,33±7,5b
1	50	44,33±6,5ac
	100	48±4ac
3	50	41±3a
	100	46.66±7,02ac
4	50	44,66±1,15ac
	100	44,66±8,5ac

** Means with the same letter do not differ statistically at the level of 0.05. SD: Standard Deviation*
***1**: 2-hydroxy-1,4-naphthoquinone, **3**: 2-phenyl-2-(2-thiophenyl)-2,3-dihydronaphtho[2,3-b]furan-4,9-dione, **4**: 2-hydroxy-3-[(E and Z)-2-phenyl-2-(2-thiophenyl)ethenyl]naphthalene-1,4-dione*

Table 3. The protective potential effects of 2-hydroxy-1,4-naphthoquinone derivatives against H_2O_2-induced genotoxic effects on and *S. cerevisiae* BY4741 *in vitro*

Test chemicals	Amount (mM)	DNA damage Abritrary Unit± SD
Kontrol	-	35,66±4,04a
H_2O_2	10	83,33±7,5b
1 + H_2O_2 (5 mM)	50	82,33±3,51b
	100	78,33±3,12be
3 + H_2O_2 (5 mM)	50	81±1,73b
	100	73,66±3,21de
4 + H_2O_2 (5 mM)	50	69,66±2,51d
	100	61,33±3,05c

** Means with the same letter do not differ statistically at the level of 0.05. SD: Standard Deviation*
***1**: 2-hydroxy-1,4-naphthoquinone, **3**: 2-phenyl-2-(2-thiophenyl)-2,3-dihydronaphtho[2,3-b]furan-4,9-dione, **4**: 2-hydroxy-3-[(E and Z)-2-phenyl-2-(2-thiophenyl)ethenyl]naphthalene-1,4-dione*

In the present study, no genotoxic effects of 1,4-naphthoquinone derivatives have been observed in yeast *S. cerevisiae* cells. No genotoxic potential of 2-hydroxy-1,4-naphthoquinones has also been demonstrated in rat hepatocyte and Chinese hamster ovary cells [32,38]. However, some naphthoquinones like 2-hydroxy-1, 4-naphthoquinone and minohydroxynaphthoquinones showed genotoxicity in B16F1 melanoma tumor cells and Chinese hamster lung fibroblasts, respectively [39]. Naphthoquinone ability to induce free radicals was strongly due to the substituents present in the quinoid molecule and by its reduction [40]. These differences in findings, suggest that different derivatives and substituents react differently and show different genotoxic potential.

3 (at 100 µg/ml) and **4** (at 50 and100 µg/ml) showed protective effects against H_2O_2. Concentration dependent relationship was seen, as higher concentrations of chemical showed a more protective effect against the DNA damage generated by H_2O_2. The mechanism of this antioxidant action of **3** and **4** should be explained. The cytotoxicity of naphthoquinones has been attributed to the production of reactive oxygen species (ROS) and electrophilic metabolites [41-43] but the capacity of ROS is dramatically affected by the position and the nature of substituents and contributes to both toxic and therapeutic actions of these substances [44]. This showed that newly synthesized naphthoquinone derivatives have chemotherapeutic response against DNA damage and can decrease the genotoxicity of damaged genetic material. According to Ramirez et al. [15] disubstituted 1,4-naphthoquinone compounds may interact with GSH to inhibit its scavenging activity of ROS. Because GSH is a pivotal molecule in inhibiting oxidative stress and acting as a scavenger for ROS and various electrophiles [45,46].

5. CONCLUSION

The novel synthesis of 2-hydroxy-1,4-naphthoquinone derivatives (**3** and **4**) did not induce any increases in revertant numbers at concentrations tested in *S. typhimurium* strains TA98 and TA100 with or without S9 mix and any DNA damage in yeast *S. cerevisiae* cells. The protective effects of 1,4-naphthoquinone derivatives was also observed at all of **4** and 100 mM concentration of **3** in yeast *S. cerevisiae* cells. It is recommended that the

molecular mechanisms involved in the genotoxicity and mutagenicity of these 2-hydroxy-1,4- naphthoquinone derivatives should be investigated in further detail.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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