EVALUATION OF ANTIOXIDATIVE, ANTIMICROBIAL AND CYTOTOXIC ACTIVITY OF THE SYNTHETIZED ARYLMETHYLENEBIS(3-HYDROXY-5,5-DIMETHYL-2-CYCLOHEXEN-1-ONE) DERIVATIVES

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Arylmethylenebis(3-hydroxy-5,5-dimethyl-2-cyclohexen-1-one) derivatives (aryl=2-hydroxynaphthyl (1) and 3,4-dihydroxyphenyl (2) have been synthesized and their structures have been elucidated. Both compounds were examined for their antioxidant, antimicrobial and cytotoxic activity. Antioxidative activities of synthesized compounds were evaluated by 2,2-diphenyl-1-picryl-hydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) methods. The microbial screening was performed by diffusion method on bacterial strains Staphylococcus aureus, Enterococcus faecalis, Bacillus subtilis and Candida albicans. Cytotoxic activity was tested on liver hepatocellular carcinoma cell line (Hep G2) by Neutral red assay. Compared to compound 2, compound 1 showed better antioxidant and antifungal activity, while compound 2 showed better antioxidant activity with IC50 of 0.0156 mM and FRAP value 50469.44 μmol/l Fe2+. Both compounds showed cytotoxic activity. Obtained results implicate the importance of arylmethylenebis(3-hydroxy-5,5-dimethyl-2-cyclohexen-1-one) derivatives as a potential antioxidant, antimicrobial and cytotoxic agents.

INTRODUCTION

Arylmethylenebis(3-hydroxy-5,5-dimethyl-2-cyclohexen-1-one) derivatives, generally known as tetraketones, are used as important precursors for organic synthesis of heterocyclic compounds, such as acridindiones, thioxanthenes, and xanthenes. These compounds have a wide range of biological activities. They have strong antioxidative potential and significant inhibitory effects on enzyme activities such as lipoxygenase, tyrosinase and protein kinase activity. Lately, derivates of tetraketones, due to their strong antioxidative activity, have received great attention as therapeutically efficient agents against disorders in which oxidative stress plays a significant role, such as inflammation, asthma and cancer.

Antioxidants compounds act to stop or inhibit the oxidation process, in low concentrations, by getting themselves oxidized. Therefore, there has been an increased interest for the use of antioxidants in medical treatments due to their capacity to prevent oxidative stress-induced damage. Synthetic antioxidants such as, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), most commonly used in the food industry, show significant side effects. Consequently, development of new, safe and more effective antioxidants is of major interest for the pharmaceutical and food industry.

Antibiotics, as products of secondary metabolites of microorganisms and fungi, are compounds used to kill or inhibit the growth of bacteria. Frequent and uncontrolled use of antibiotics results in resistance in bacterial strains, so there is a real need for the development of new effective and antibacterial agents. Recent studies of tetraketones show that different such derivates show antibacterial as well as antiviral activity.

Previous studies of some xanthenes indicate that xanthenes have shown cytotoxic activity against human Colo-205. Another study confirmed the antiproliferative activity of xanth-3-one derivates against different human cell lines including SW620, HepG2, HeLa and A549 tumor cells. Thioxanthenes and xanthendiones are formed as products of cyclization of tetraketone by nucleophilic addition of hydroxyl group -OH to the C=C bond, therefore, their biological activity may be related to the formation of these compounds.

The aim of this study was to synthesize two new biologically active tetraketones via Knoevenagel condensation and Michael addition of benzaldehydes with dimedone and catalyst, and to evaluate their antioxidative and antimicrobial activities, as well as to assess their cytotoxic effect on human liver hepatocellular carcinoma cell line (HepG2).

EXPERIMENTAL

The chemicals used in the synthesis of arylmethylenebis (3-hydroxy-5,5-dimethyl-2-cyclohexen-1-one) derivatives were obtained from the Merck (Darmstadt, Germany) and Sigma Aldrich (Missouri, SAD). All analyses were performed using analytical grade chemicals, reagents and standards. Neutral red assay kit was obtained from Sigma Aldrich (Missouri, SAD). For all dilutions, only double- distilled deionized water was used. Melting points of compounds were determined by capillary method on Kruss Melting point KSP I. IR spectra of synthesized compounds were recorded by Thermo Scientific Nicolet iS10 FTIR Spectrophotometer. The H...
and $^{13}$C nuclear magnetic resonance (NMR) spectra were recorded at 500 and 150 MHz in deuterated acetone at 25 °C using NMR spectrophotometer Bruker Avance III 500 (500 MHz for $^1$H, 125 MHz for $^{13}$C). Elemental microanalyses of synthesized compounds were performed by ELEMENTAR (C, H, N, S, O) VARIO EL III. Antioxidant activities were recorded by Spectrophotometer UVmini-1240, Shimadzu Corporation.

General procedure for the synthesis

A mixture of 5,5-dimethylcyclohexyne-1,3-dione (2 mM), substituted benzaldehyde (1 mM) and DABCO (10 mM) in water (20 mL) was refluxed for 30 min. After completion of the reaction, the mixture was cooled to the room temperature. The solid was filtered off, washed with distilled water and recrystallized from 96 % ethanol.

Determination of antioxidant activity

2,2-Diphenyl-1-picrylhydrazyl (DPPH) method was performed as described earlier by Lee et al. The radical scavenging effect (%) or percent inhibition of DPPH radical was calculated according to the equation:

$$\% \text{ inhibition} = \left( \frac{A_{\text{sample}} - A_{\text{control}}}{A_{\text{control}}} \right) \times 100$$

where $A_{\text{sample}}$ is the absorbance of the solution containing the sample at 517 nm and $A_{\text{control}}$ is the absorbance of the DPPH solution. The results are expressed as the IC$_{50}$ value (mM) or the concentration of the sample that caused 50 % neutralization of DPPH radicals.

The determination of ferric reducing antioxidant power or ferric reducing ability (FRAP assay) was performed by the method described by Pirvu et al. The standard solutions of FeSO$_4$·7H$_2$O were prepared in the concentration range from 200 to 1000 μmol L$^{-1}$ ($y = 0.001x + 0.0615; R^2 = 0.9907$). In each tube, 0.1 mL of sample and 3 mL of FRAP reagent were added. The samples were incubated in an aqueous bath for 30 min at 37 °C, and the absorbance was measured at 593 nm.

Determination of antimicrobial activity

Antimicrobial activity was determined using the diffusion method described by Pirvu et al., on reference bacterial strains from Gram-positive microorganisms (Staphylococcus aureus ATCC 25923, Enterococcus faecalis ATCC 51299, Bacillus subtilis ATCC 23857). Antifungal activity was examined on Candida albicans (ATCC 90028). From the bacterial strains of overnight cultures, suspensions of 0.5 McFarland turbidity were prepared (density $10^7$-$10^8$ CFU mL$^{-1}$, depending on soy). The strains were placed then on the surface of the nutrient substrate-Mueller-Hinton agar (MH), dispersed in sterile Petri dishes. The substrate thickness was 4 mm. In the agar, sterile drill-shaped holes were made ("wells"), into which 50 μL of synthesized compounds (1-2) in concentration of 2 mg mL$^{-1}$ were added. After the plates were left at room temperature for 15 min to the substance was diffused into agar, they were incubated at 37 °C for 24 h. After the incubation period, the size of the inhibitory zone was measured and the sensitivity of the microorganisms was expressed as follows. If the zone for inhibition of microorganism growth was greater than 20 mm, it was labeled with three pluses (++++), representing the highest sensitivity of the microorganisms. If the inhibitory zone ranged from 16 to 20 mm, it was marked with two pluses (++). If the inhibitory zone was 10-15 mm in diameter, it was marked with a plus (+). For the inhibitory zone, less than 10 mm or if absent, a minus (-) sign has been used.

In vitro determination of cytotoxic activity

The liver hepatocellular carcinoma cell line was cultured in Minimum Essential Medium Eagle medium (Sigma Aldrich) containing 2 mM glutamine (Sigma Aldrich), 1 % nonessential Amino Acids (Sigma Aldrich), 10 % heat-inactivated (HI) FBS (Sigma Aldrich) and 1 % penicillin/streptomycin antibiotics (Sigma Aldrich), in a humidified atmosphere containing 5 % CO$_2$ at 37 °C. For each experiment, cells were grown at 80 % confluence in 75 cm$^2$ culture flasks.

Cytotoxic activity (cell viability) was evaluated by Neutral red assay using the method previously described by Repetto et al. For each experiment, HepG2 cells were seeded (2 x 10$^4$ cells well$^{-1}$) in 96 well plates and grown for 24 h. Test agents (2.5 µM-100 µM) were then added and cells were incubated for an additional 24 h. Working dilutions were freshly prepared on the day of testing in the growth medium. The solvent (DMSO) concentration never exceeded 0.1 % in the final medium containing cells. Untreated cells were used as negative control and positive control were cells treated with 5-fluorouracil (5-FU). Cells were treated with 10 % compound solutions and 90 % culture medium. Compound stock solutions were prepared in double-distilled deionized water and solutions were sterilized by filtration through 0.2 μm sterile syringe filters.

The Neutral red assay was carried out according to the manufacturer's instructions (Sigma Aldrich). After 24 h treatment cells were incubated with medium containing neutral red dye for 3 h. The cells were subsequently washed, the dye was extracted from cells and absorbance was measured using a microplate reader at a wavelength of 540 nm. The measured absorbance values were converted to percent of cell viability with respect to negative control.

RESULTS AND DISCUSSION

Synthesis of arylmethylenbis(3-hydroxy-5,5-dimethyl-2-cyclohexen-1-one) derivatives

As shown in Figure 1, the synthesis of arylmethylenbis(3-hydroxy-5,5-dimethyl-2-cyclohexen-1-one) was assumed to proceed via forming a product of Knoevenagel condensation and Michael addition. In the first step of the synthesis, there is a condensation of 5,5-dimethylcyclohexane-1,3-dione and the 2-naphthylaldehyde and 3,4-dihydroxyphenylaldehyde. After dehydration and Michael's addition, synthetized tetraketone converts to arylmethylenebis(3-hydroxy-5,5-dimethyl-2-cyclohexen-1-one) derivative via keto-enol tautomerism.
Arylmethylene-3-OH-5,5-Me2-2-cyclohexen-2-ones  

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Figure 1. Mechanism of synthesis of 2,2'-arylmethylen-bis(3-hydroxy-5,5-dimethly-2-cyclohexen-1-one) derivatives.

2.2'-(2-Hydroxynaphtyl)methylenebis(3-hydroxy-5,5-dimethyl-2-cyclohexen-1-one) (1)

Yield 98.80 %, m.p. 236-240 °C. IR: 3200 (O-H), 3000 (Ar-H), 2934 (Aliph. -CH), 1641 (C=O), 1590 and 1465 (C=C), 1369 (C-O), 1230 (˗OH) cm⁻¹. 1H NMR (500 MHz): δ = 0.52-0.80 (4s 12H, C(CH₃)₂); 1.70- 2.10 (dd, 4H, CH₂); 2.02-2.26 (dd, 4H, CH₂); 5.46 (s1H CH); 7.19 -8.02 (6 d, 6H, Ar-H); 11.09 (s,1H, -OH). 13C NMR δ = 26.5-30.0 (CH₃ on C5 and C5'); 30.07 (C5 and C5'); 32.2 (C 7); 41.6, 43.7 (C 4 and C4'); 50.3, 51.3 (C and C'); 50.3, 51.3 (C and C'); 111.9 (C2 and C2'); 117.4, 132.2, 149.9 (Ar-C); 115.5, 123.9, 125.2, 127.4, 129.2, 120.4 (Ar-CH); 195.5, 200.9 (C1 and C1'); Anal. Calcd for C₂₇H₃₀O₅: C 74.63, H 6.96. Found C 74.98, H 6.59.

2,2'-(3,4-dihydroxyphenyl)methylenebis(3-hydroxy-5,5-dimethyl-2-cyclohexen-1-one) (2)

Yield 53.50 %, m.p. 196.5-198.5 °C. IR: 3236 (O-H), 3000 (Ar-H), 2966 (Aliph CH), 1652 (C=O), 1558 and 1467 (C=C), 1369 (C-O), 1249 (OH) cm⁻¹. 1H NMR: δ = 1.14 (s, 12H, C(CH₃)₂);  2.12 (s, 4H, CH₂); 2.38 (s, 4H CH₂); 5.41 (s, 1H, CH); 6.64; 6.69 (dd, 3H Ar -H); 7.67; 7.69 (s, 2H, OH); 12.01 (s, 1H, OH). 13C NMR: δ = 28.6 (CH₃ on C5 and C5'); 32.2 (C5  and C5'); 33.0 (C7); 47.3 (C₄ and C₄'); 115.5, 115.9, 119.2, 131.0 (Ar-CH) 144.0, 145.8 (Ar-C); 116.5 (C2 and C2'); 190.4 (C1, C3 and C1', C3'). Anal. Calcd for C₂₃H₂₈O₆: C 68.98, H 7.05. found C 68.77, H 6.82.

Structural characterization of compounds

The IR spectrum indicated that both samples show absorption bands for tetraketone structure, stretching vibrations between 3000-2900 cm⁻¹ came from aliphatic CH group, absorption bands at 1700-1600 cm⁻¹ belong to C=O groups. Further, all IR spectrum show bands at 1600 and 1446 cm⁻¹, which are characteristic for the aromatic ring (C=C), there are bands at 1300 cm⁻¹, characteristic for C-O stretching and deformation vibrations of the OH group at 1200 cm⁻¹. The aromatic hydroxy groups as substituents in the spectrum of compound 1 with 2-hydroxynaphyl group show a characteristic band at 3200 cm⁻¹, while the spectrum of compound 2 with two hydroxy groups on phenyl ring on the positions of 3 and 4, has a characteristic band at 3236 cm⁻¹.

The 1H NMR spectra of synthesized compounds contain broad singlets in the range of 1.14-1.18 ppm derived from CH₃ protons belong to position s C5 and C 5'. Both spectra have 2-2 singlets in the range of 2.03-2.12 ppm and 2.38 -2.41 ppm due to CH₂ protons on positions of C4, C6, and C4', C6'. One singlet was observed at 5.41- 5.46 ppm from CH proton, which connects cyclohexene rings (C7).

A singlet at 4.29 ppm (proton of the hydroxy group) and three doublets in the range of 7.19- 7.36 ppm and doublets in the range of 7.26-8.02 derived from the protons of the naphthyl group could be observed in the spectrum of compound 1. Two doublets were found at 6.64-7.52 ppm due to phenyl ring protons of compound 2, while the hydroxy group signals had a singlet at 6.97-7.67 ppm

The 13C NMR spectra of synthesized compounds showed signals at 26.5-29.0 ppm belong to carbon atoms of the methyl groups. The signals from carbons on positions C5 and Cr are at 30.07-32.8 ppm, while the signal from the methine group connects the two cyclohexene rings that appeared at 32.2-33.6 ppm. Signals characteristic for cyclohexene ring appeared at 47.6-51.3 (C₄, C₆ and C₆', C₈), 111.9-116.6 (C₂ and C₂') and 190.4-200.9 ppm (C₁, C₃ and C₁', C₃'). In the 13C NMR spectra, signals for aromatic carbons appeared at 117.4-125.2 and 127.4-132.2 ppm or 112.2-129.0 and 131.0-150 ppm for compound 1 and compound 2, respectively.

Figure 2. Structure of synthesized compounds, 1 and 2.
Antioxidant activity

The results of antioxidant activity expressed as \( IC_{50} \) and FRAP value, are shown in Table 1. Results indicated 2 has the strongest free radical scavenging effect with \( IC_{50} \) of 0.0156 mM and a FRAP value 50469.44 µmol L\(^{-1}\) Fe\(^{2+}\), which may be due to the formation radical on the hydroxy group (substituted on phenyl ring in positions 3 and 4). Compound 1, substituted with two hydroxynaphthyl groups, has a free radical scavenging effect and \( IC_{50} \) of 1.79 mM and FRAP value of 290.95 µmol L\(^{-1}\) Fe\(^{2+}\).

Table 1. Summarized results of antioxidant capacity.

| Sample  | \( IC_{50} \) value, µmol L\(^{-1}\) | FRAP value, µmol L\(^{-1}\) Fe\(^{2+}\) |
|---------|----------------------------------|-----------------------------------|
| 1       | 1.790                            | 290.95                            |
| 2       | 0.015                            | 50469.44                          |
| Vitamin C | 0.035                           | 14250                             |

In comparison with Vitamin C, compound 2 exhibited higher antioxidant capacity. These results suggest that hydroxy groups have a significant effect on the antioxidant activities of synthetic antioxidant substances. It has been previously reported that reducing power is associated with antioxidant activity, which is in accordance with our findings.\(^{30}\) Further, significant effects of hydroxy group position on antioxidative activity have been previously reported.\(^{3} \) Also, a good antioxidant activity of other tetraketones in the literature has been reported, which correlates with our results.\(^{31}\)

Antimicrobial activity

Results of the in vitro antimicrobial activity of the synthesized compounds against selected test microorganisms are shown in Table 2. Results indicate that compound 1 has antibacterial activity against all tested Gram-positive bacteria, and has antifungal activity against Candida albicans. The largest inhibition zone, for compound 1, was recorded with Staphylococcus aureus (20 mm) and the smallest with Enterococcus faecalis (10 mm). Compound 2 has antimicrobial activity only against Staphylococcus aureus (11 mm). In comparison to compound 2, compound 1 showed more potent antimicrobial and antifungal activity. This probably occurs due to the presence of 2-naphthol group in the structure of compound 1. According to the literature the naphthalene ring, as a potent lipophilic part of the structure,\(^{32}\) better passes through biomembranes and therefore acts as a potent antimicrobial agent.\(^{33}\)
Cytotoxic activity

Assessment of the cytotoxic potential of tetraketones on human-derived carcinoma cell line is the subject of many scientific studies.34 Our findings suggest that both of our samples are potent cytotoxic agents (Figure 3). As expected, compound 1 exhibited higher cytotoxic potential, causing a higher percent of cytotoxicity at the same concentration compared to compound 2. It was interesting that the most pronounced cytotoxic effect was observed at the highest concentrations of compound 2 (100 µmol L⁻¹). It is noticeable that the increase in concentrations necessarily leads to a proportional decline in cell viability in both cases, respectively. These results are in agreement with previously reported data which suggest that concentrations of cytotoxic substance are in direct relationship with cell viability.21

CONCLUSION

Two arylmethylenebis(3-hydroxy-5,5-dimethyl-2-cyclohexen-1-one) (aryl = 2-hydroxynaphthyl (1) and 3,4-dihydroxy-phenyl (2) derivates were synthesised and tested for antioxidant, antimicrobial and cytotoxic activities. From the results, we can conclude that better antioxidant activity with IC₅₀ of 0.0156 mM and FRAP value 50 469.44 µmol L⁻¹ Fe²⁺ has been found for compound 2, while better antimicrobial and antifungal activity could be found for compound 1. Although both compounds showed cytotoxic activity, compound 1 exhibited higher cytotoxic potential in comparison to compound 2. The results obtained showed significant antioxidant, antimicrobial, and cytotoxic activities which support the importance of these compounds as candidates for therapeutically efficient agents against oxidative stress, microorganisms, and tumor cells.

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Table 2. Antimicrobial activity of compounds in diameters, in mm, of inhibition zone.

| Sample | SA | EF | BS | CA |
|--------|----|----|----|----|
| Compound 1 | 20 (++) | 10 (+) | 13 (+) | 14 (+) |
| Compound 2 | 11 (+) | - | - | - |
| Ciprofloxacin | >25 (++) | >25 | >25 | (++) |
| Nystatin | - | - | - | 20 (++) |

Legend: SA - S. aureus, EF - E. faecalis, BS - B. subtilis, CA - Candida albicans.
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