

Electrochemical detection of antioxidant activities of 4-indolyl-5-oxo-6,6 (or 7,7) -dimethyl-1,4,5,6,7,8-hexahydroquinoline derivatives

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ABSTRACT: Antioxidants used in different medical and industrial fields in order to prevent and delay oxidative stress. They play a crucial role in the protecting biological systems against many diseases. 1,4-dihydropyridines are known as calcium channel modulators. Electrochemical techniques are simple, cheap and fast detection techniques and require small amounts of sample, so they offer advantages over commonly used analytical methods. Voltammetric methods have been applied to investigate the antioxidant activity of compounds in different fields. The proposed work is aimed at examining the electrochemical behavior of the 1,4-dihydropyridines by differential pulse voltammetry and hence the assessment of its antioxidant activity from the cathodic reduction peak of oxygen values. The peak current due to oxygen reduction was found to be proportional to the 1,4-dihydropyridines concentration of 0.1 - 0.5 mg/mL. The coefficient of antioxidant activity of 1,4-dihydropyridine derivatives were calculated and compared each other. Nifedipine used as a reference drug that is known as the calcium channel modulator and it is used to compare the antioxidant activities of 1,4-dihydropyridine-derived compounds.

KEYWORDS: Hexahydroquinoline derivatives; nifedipine; antioxidant activity; differential pulse voltammetry.

1. INTRODUCTION

Antioxidants have been used in many industry and medicine fields to prevent and delay oxidative stress in the recent years. They play a very important role in the protecting biological systems against many diseases[1]. Free radicals are responsible for the appearance of different diseases such as diabetes, neurological and cardiovascular diseases and different types of cancer. Antioxidants can stop oxidation reactions that caused by these free radicals associated with DNA degradation, protein denaturation and membrane peroxidation [2-4].

Oxidative stress is formed by the action of reactive oxygen species (ROS) and is acted in different diseases [5, 6]. ROS, such as superoxide anion radical (O_2^-) singlet oxygen (O_2), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH) are formed in organism by metabolism, inflammatory reactions or environmental factors [7]. They affect DNA, proteins and lipids generating many tissue damages. Antioxidants are think that prevent the negative effects of ROS and the regulate the defense systems and therefore could be used for prevention and treatment of oxidative stress-related diseases [6, 8].

1,4-Dihydropyridines (1,4-DHPs) (Figure 1) are known as calcium channel modulators. 1,4-DHPs are the appropriated heterocyclic ring with various substitutions at different positions. 1,4-DHPs inhibit Ca^{2+} influx provides an important therapeutic healing in the treatment of angina pectoris, hypertension and muscle diseases. The most appropriate position for substitution is the 4th position which shows diverse calcium channel antagonist effects and the heterocyclic ring is the general characteristic for antianginal, analgesic, anti-inflammatory, antihypertensive, antitubercular, antithrombotic, antitumor and antioxidant activity [9-15]. The working 1,4 DHPs derivatives are shown in Table 1.

Nifedipine (NIF), a calcium channel blocker, is 1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridine-dicarboxylic acid dimethyl ester. It is used for the treatment of arterial hypertension and angina pectoris [16, 17].

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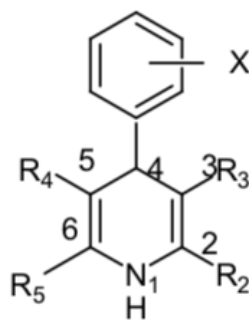


Figure 1. Chemical structure of 1,4 DHPs.

Table 1. The structures of the 1,4 DHPs compounds.

R	A Group	B Group	C Group	D Group
Compound No				
CH ₃	1	2	3	4
C ₂ H ₅	5	6	7	8
CH(CH ₃) ₂	9	10	11	12
CH ₂ CH(CH ₃) ₂	13	14	15	16
C(CH ₃) ₃	17	18	19	20
CH ₂ C ₆ H ₅	21	22	23	24

Voltammetric methods are among the most common electrochemical methods. A specific voltage is applied to a working electrode as a function of time and the current is measured in voltammetric techniques. Differential pulse voltammetry (DPV) is the most widely used in the voltammetric methods [18, 19]. The potential is changing linearly with the time and added by the potential pulses of the amplitude for several milliseconds in this method. Peak current is subtracted from the peak current measured at the end of a potential step and the peak current measured before the step. The peak current depends on the concentration of the studied analytes [20-23].

Many analytical methods have been used to evaluate the antioxidant activity in pharmaceuticals, clinical biology, cosmetics and food areas [24-26]. Voltammetric techniques supply high potential for assessment of antioxidant properties of different compounds. Different electrodes such as solid electrodes can be used for in these analysis [27-30]. These methods are fast, simple, inexpensive and sensitive methods for analysis of antioxidant compounds. Cyclic voltammetry [31, 32], differential pulse voltammetry [26, 33-35], square wave voltammetry [36, 37] and amperometry [38, 39] are used in these analyses.

The single-use disposable electrodes are frequently used in electrochemical analysis and they may solve the regeneration disadvantages of the solid electrodes. Pencil leads are important electrode materials with good mechanical stability and high electrochemical reactivity, and cheap [40, 41]. Due to their properties, pencil graphite electrodes (PGE) have been used frequently in the electrochemical analysis of various organic and inorganic materials from different mediums in recent year [42-44]. The sensing area of the pencil can be easily controlled and readily disposed of after use. Solid electrodes involve tedious surface polishing steps. When compared to other working electrodes, PGE show strong adsorption properties, wide potential window, low background current and ease to use [45-48].

Antioxidant activity of compounds is determined by measuring the oxygen reduction peak current using DPV method. The process is based at the cathode with generation of the active anion-radical of oxygen, superoxide (O₂⁻) [49-51]:



The initial stage in this process is the one-electron reduction of oxygen by the generation of active oxygen radicals O_2^- and $\text{HO}_2 \cdot$. Antioxidants of the reductive nature react with these radicals on the surface of the working electrode, which leads to a decrease of current of the one-electron reduction of oxygen on the PGE [49, 50].

This proposed study is aimed at studying the electrochemical behavior of the 1,4 DHPs and NIF by a voltammetric technique as DPV and hence the assessment of antioxidant activity of these compounds from the cathodic peak values. The antioxidant activity of some antioxidants as 1,4 DHPs and NIF were evaluated using reduction of oxygen molecule and the voltammograms were scanned in the potential range between $E = 0.0 \text{ V}$ and -2.0 V vs. Ag/AgCl reference electrode.

2. RESULTS AND DISCUSSION

Many different methods such as spectrophotometry [26, 52-54], fluorometry [52], high-performance liquid chromatography (HPLC) [26, 55-57] and capillary electrophoresis [55, 58] in combination with different detectors have been used to investigate of the antioxidant activity of different compounds.

In spectrophotometric studies, the antioxidant activities of phenolic compounds determined by the TEAC (Trolox equivalent antioxidant capacity), DPPH (using diphenyl-p-picrylhydrazyl radical), and FRAP (ferric reducing antioxidant power) methods. In fluorometric method, antioxidant activity of compounds determined by the ORAC (oxygen reactive antioxidant capacity) method. In addition, cupric ion reducing antioxidant capacity (CUPRAC) analysis method was used in these analysis [59].

Scanning electrochemical microscopy (SECM) was applied to evaluate the oxidation reaction mechanism and kinetic parameters of antioxidants and at oil/water or liquid/liquid interfaces. [60, 61].

Voltammetric techniques are considered to be the most effective methods for evaluating the antioxidant properties of compounds. These methods are based on the decrease of the electro-reduction oxygen peak current in the existence of the studied antioxidant species [51, 62].

Electrochemical behaviors of 1,4-DHPs derivatives and NIF were examined by DPV technique. 1,4-DHPs derivatives and NIF were dissolved in 0.1 M tetrabutylammonium iodide [TBAI, $(\text{C}_4\text{H}_9)_4\text{NI}$] salt - dichloromethane (DCM) solution. DCM was used as chlorinated solvents and its non-bonding structure and lack of Lewis base properties made DCM a suitable solvent in electrochemical analysis [63]. TBAI is indicate excellent stability of cations and easily purified in DCM solvent and it is ideal for the reductions of 1,4-DHPs derivatives and NIF [64, 65].

The DPV response of the studied 1,4 DHPs derivatives depend on the instrumental parameters. Different parameters influencing the peak current intensity and shape of the peak of studied derivatives were investigated in order to improve sensitivity and repeatability. In order to obtain the maximum peak current and the best peak shape for 1,4 DHPs derivatives, the instrumental parameters such as the pulse amplitude (A), pulse increment (E_s), pulse time (W) and pulse period (T) parameters of proposed DPV method were investigated. A (20 - 50 mV), E_s (10 - 30 mV), W (0.1 - 0.3 s) and T (0.3 - 0.5 s) were studied and optimized. Consequently, optimized parameters were 50 mV, 20 mV, 0.1 s and 0.5 s, respectively in the analysis.

After the solution was stirred for a certain time, stirring was stopped. The potential was scanned negatively and a peak was obtained to causing oxygen reduction. This peak current value is related to the amount of oxygen in the solution.

The DP voltammograms of oxygen reduction in 0.1 M TBAI - DCM solution including 1,4 DHPs derivatives and NIF (0.1 mg/mL) at the PGE were recorded. It showed that in the cathodic direction a well - defined oxygen reduction voltammetric peak between $-789.5 - (-629.7) \text{ mV}$ potential range vs. Ag/AgCl electrode (Figure 2).

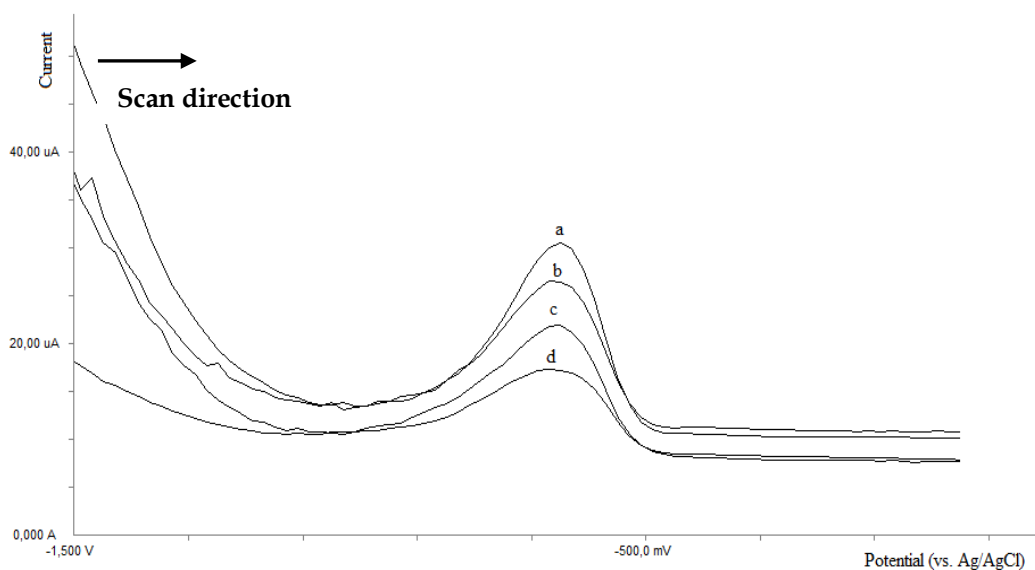


Figure 2. DP voltammograms of oxygen reduction peaks for different concentrations of compound 10, using the experimental conditions. (a) Supporting electrolyte, (b) 0.1, (c) 0.3 and (d) 0.5 mg/mL in 0.1 M TBAI - DCM solution.

At first in this study, a voltammogram without antioxidant was recorded to obtain the original limiting value of the oxygen peak current (I_{or}) due to the reduction of oxygen in the 0.1 M TBAI - DCM solution as used supporting electrolyte. Dissolved oxygen was extracted from the supporting electrolyte solution by a nitrogen gas through the electrochemical cell for sufficient period. The voltammogram of the supporting electrolyte lowest oxygen was taken to obtain the residual current value (I_{res}).

After that, an antioxidant solution of 1,4 DHPs in the linear concentration range was added to the electrochemical cell containing supporting electrolyte and the proportional decrease of the oxygen current related to the concentration of the added studied antioxidant at certain potential was observed (I_{res}). Voltammograms were recorded in a wide potential range ($E = 0$ to -2.0 V). It was shown that 1,4 DHPs did not exhibit electrochemically activity under the experimental conditions.

Due to the highest oxygen content of the electrolyte solution, the highest cathodic peak current (I_{or}) was obtained and the nitrogen gas passed solution which showed the lowest cathodic current (I_{res}) because of the lowest oxygen content.

The graphs of the relative change of the oxygen reduction current density ($j / (j_{or} - j_{res})$) against studied antioxidant concentration (mg/mL) in the 0.1 M TBAI - DCM solution was plotted (Figure 3). The slope of these lines was accepted as a coefficient of antioxidant activity (K) of the 1,4 DHPs and NIF that was calculated according to the formula and the calculated K values are given in Table 2 [49, 51]:

$$K = \frac{\Delta j}{(j_{or} - j_{res}) \Delta c} \quad [\text{Eq. 5}]$$

Δj : Change of the oxygen reduction current density with the additions of 1,4 DHPs and NIF

Δc : The change of the antioxidant concentration (mg/mL)

j_{or} : The limiting current density of the oxygen reduction without antioxidants in the solution

j_{res} : The residual current density after removal of oxygen by nitrogen gas in the solution under constant potential.

In order to verify the method linearity between oxygen reduction peak currents and 1,4 DHPs / NIF concentrations, calibration curves were plotted using standard solutions at different 3 concentration levels under the optimum conditions and 0.1 MTBAI - DCM solution. The peak current of oxygen reduction was found to be directly proportional to the 1,4 DHPs / NIF concentration between 0.1 and 0.5 mg/mL. The oxygen peak currents were linearly decreased with increasing concentration of 1,4 DHPs / NIF. Least - squares regression analysis was performed for the obtained data and the results are given in Table 2.

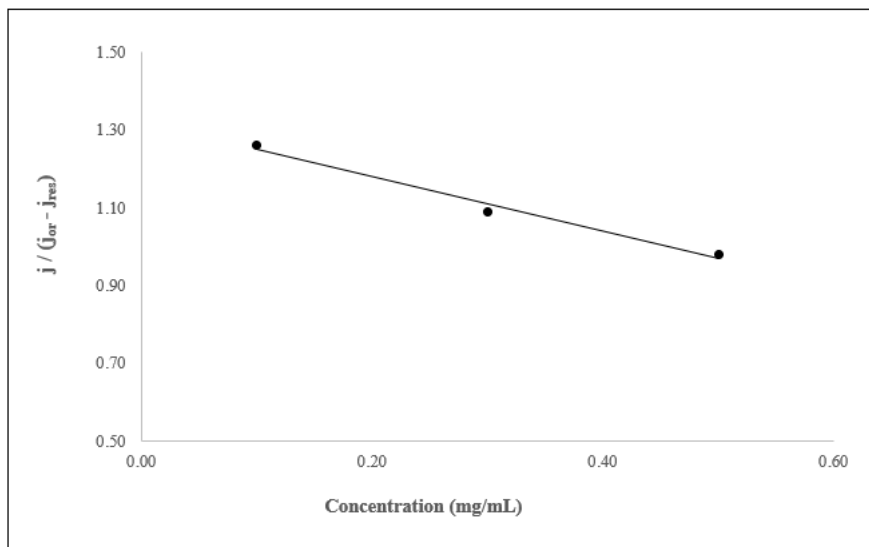


Figure 3. Curve of the relative change of the oxygen reduction current density against 1,4 DHPs concentration in the 0.1 MTBAI - DCM solution for compound 3.

Electrochemical behaviors of 1,4-DHPs derivatives and NIF were investigated using CV and DPV techniques in the literature [63]. The obtained results of antioxidant activities were compared with the results of NIF. The coefficient of antioxidant activity was obtained between 0.50 - 0.79 for 1,4-DHPs and 0.72 for NIF, respectively. In our study, the coefficient of antioxidant activity was calculated as between 0.50 - 0.70 for 1,4-DHPs and 0.68 for NIF. These calculated antioxidant activity values of 1,4 DHPs and NIF in our study corresponds to the value given in the literature [63].

Different K values were obtained on the surface disposable PGE due to different structures of 1,4 DHPs and NIF. The K values found from the formula and the relative change in the oxygen reduction current density were the same. All the substances being studied showed varying antioxidant activity. Antioxidant activities of the 1,4-DHPs derivatives in A - B and C - D groups were found to be close to each other. Antioxidant activities of 1,4-DHPs and NIF were also found to be close to each other. The antioxidant activity coefficients of the 1,4 DHPs derivatives in A and B groups were found to be higher than those in C and D groups on the basis of oxygen reduction peak current. This difference between groups A - B and C - D may be thought to be due to Br's position in indole ring. In the literature, it has been stated that compounds with a large K value have higher antioxidant activities [34, 49, 50, 51, 63]. Therefore, it can be said that antioxidant activities of A and B groups are higher than other studied 1,4-DHPs derivatives. There was no significant difference in antioxidant activities of groups A, B, C and D due to different R substituents.

3. CONCLUSION

Voltammetric methods have been demonstrated to be sensitive for the determination of organic molecules, including drugs and different molecules in pharmaceutical formulations. These techniques have several advantages, including that they have low running costs, the use of less expensive, simpler, faster compared with the spectrophotometric and chromatographic methods. Therefore, voltammetric methods are used widely the reduction or oxidation-related analysis of organic or inorganic compounds using different electrodes.

DPV method used in this study is very simple. The simple sample preparation in the proposed DPV method was described as dissolving the standard and adequate dilution by the supporting electrolyte. This method based on the scanning of voltammograms of the reduction of oxygen molecule related to studied compounds and also small amounts of antioxidants were found sufficient for analysis. PGEs are used by their low cost, disposability, simplicity and commercial availability.

Table 2. Antioxidant activity coefficients for 1,4 DPHs derivatives and NIF.

Compound	Reduction Potential (V)	*K _{av}	Average regression equation ^a	r value
1	-729.6	0.63	y = - 0.6303x + 1.3563	r = 0.9678
2	-729.6	0.68	y = - 0.6766x + 1.3967	r = 0.9764
3	-729.6	0.62	y = - 0.6250x + 1.4309	r = 0.9744
4	-789.5	0.66	y = - 0.6625x + 1.3938	r = 0.9780
5	-729.7	1.23	y = - 1.2203x + 1.2350	r = 0.9897
6	-709.6	0.94	y = - 0.9335x + 1.3495	r = 0.9549
7	-689.6	0.69	y = - 0.6875x + 1.5596	r = 0.9833
8	-749.6	0.63	y = - 0.6125x + 1.5304	r = 0.9893
9	-669.6	0.58	y = - 0.5828x + 1.4109	r = 0.9808
10	-649.6	1.54	y = - 1.5424x + 1.4815	r = 0.9958
11	-669.6	0.50	y = - 0.5000x + 1.3550	r = 0.9989
12	-689.7	0.49	y = - 0.5000x + 1.4684	r = 0.9883
13	-629.8	1.71	y = - 1.7092x + 1.2418	r = 0.9867
14	-629.7	1.54	y = - 1.5463x + 1.5744	r = 0.9965
15	-629.8	0.69	y = - 0.6878x + 1.1945	r = 0.9893
16	-689.7	1.09	y = - 1.0919x + 1.4978	r = 0.9895
17	-669.7	0.71	y = - 0.7050x + 1.2437	r = 0.9577
18	-649.7	0.90	y = - 0.8997x + 1.4436	r = 0.9879
19	-629.7	0.27	y = - 0.2730x + 1.1619	r = 0.9784
20	-629.7	0.23	y = - 0.2297x + 1.2120	r = 0.9866
21	-689.7	0.51	y = - 0.5067x + 1.9086	r = 0.9769
22	-729.7	1.54	y = -1.5387x + 1.7803	r = 0.9790
23	-689.6	0.68	y = -0.6849x + 1.4175	r = 0.9924
24	-689.6	0.58	y = - 0.5781x + 1.5862	r = 0.9652
NIF	-709.7	0.68	y = - 0.6744x + 1.4439	r = 0.9912

^ay = bx + a; x = concentration (mg/mL), y = peak current (µA), a = intercept, b = slope.

In this study, antioxidant activities of 1,4-DHPs derivatives were evaluated by using DPV method from the reduction peak current of oxygen on the disposable PGE surface. The coefficient of antioxidant activity (K) of 1,4-DHPs derivatives were calculated and compared with each other and NIF.

The antioxidant activity coefficients of the 1,4-DHPs derivatives in groups A and B were found to be higher than those in groups C and D on the basis of oxygen reduction peak current. There was no significant difference in antioxidant activities of 1,4-DHPs derivatives due to different R substituents.

4. MATERIALS AND METHODS

4.1. Materials

NIF was kindly supplied by Fako A.S. and it was used without further purification. All other chemicals were of reagent analytical grade quality. TBAIsalt was purchased from Sigma and it was dissolved in DCM solvent. 3 mL of DCM solvent containing 0.1 M of TBAI was used as a supporting electrolyte.

4.1.1. Synthesis of Alkyl 4-(5/6-bromo-1H-indole-3-yl)-2,6,6/2,7,7-trimethyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylates

The 1,4-DHP derivatives (Compound 1-20) with antioxidant activity have been synthesized by Baydar, E. et. al. and their structures have been proven by instrumental techniques. This study was previously published [66]. Compounds 21 - 24 were the original compounds synthesized for the first time.

Compounds 21 - 24 were synthesized according to the general synthesis method given in the literature for other compounds. According to this; 2 mmol diketone derivative (4,4-dimethylcyclohexane-1,3-dione or 5,5-dimethylcyclohexane-1,3-dione), 2 mmol substituted indole carbaldehyde (5-bromo-1H-indole-3-carbaldehyde or 6-bromo-1H-indole-3-carbaldehyde), 2 mmol benzyl acetoacetate and 10 mmol ammonium acetate in ethanol were reacted in 35 mL vial in microwave synthesis unit. It was subjected to microwave

irradiation at a maximum of 150 °C for 5 min. The progress of the reaction was followed by TLC. Upon completion, the reaction mixture was poured into ice-water and the resulting solid crystallized from the ethanol-water mixture [66]. The structure of the synthesized compounds was proved by ¹H-NMR, ¹³C-NMR and elemental analysis.

Benzyl 4-(5-bromo-1H-indole-3-yl)-2,6,6-trimethyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (Compound 21): Yield 74%. m.p. 90 °C. ¹H-NMR (δ ppm, DMSO-d₆) 0.87 (3H; s; 6-CH₃), 0.97 (3H; s; 6-CH₃), 1.66-1.74 (2H; m; H-7), 2.25 (3H; s; 2-CH₃), 2.48-2.51 (2H; m; H-8), 3.92-4.02 (2H; m; OCH₂), 5.07 (1H; s; H-4), 6.88-7.73 (9H; aromatic protons), 9.16 (1H; s; HHQ N-H), 10.85 (1H; s; indole N-H). ¹³C-NMR (δ) 18.6, 18.8, 18.9, 24.1, 25.3, 34.0, 39.5, 40.1, 69.0, 103.0, 108.0, 112.9, 113.6, 120.8, 121.2, 121.5, 123.0, 124.2, 127.4, 127.9, 128.9, 129.0, 136.4, 137.1, 145.3, 149.0, 167.4, 199.2. Analysis for C₂₈H₂₇BrN₂O₃: Calculated C: 64.74, H: 5.24, N: 5.39; Found C: 64.68, H: 5.43, N: 5.34.

Benzyl 4-(5-bromo-1H-indole-3-yl)-2,7,7-trimethyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (Compound 22): Yield 69 %. m.p. 102 °C. ¹H-NMR (δ ppm, DMSO-d₆) 0.78 (3H; s; 7-CH₃), 0.97 (3H; s; 7-CH₃), 1.89 (1H; d; J: 16 Hz; H-8a), 2.05 (1H; d; J: 16 Hz; H-8b), 2.23 (3H; s; 2-CH₃), 2.43 (1H; d; J: 16.0 Hz; H-6a), 2.36 (1H; d; J: 16.0 Hz; H-6b), 3.99-4.04 (2H; m; OCH₂), 5.04 (1H; s; H-4), 6.91-7.58 (9H; aromatic protons), 9.06 (1H; s; HHQ N-H), 10.75 (1H; s; indole N-H). ¹³C-NMR (δ) 18.4, 18.6, 18.9, 22.1, 26.8, 33.9, 39.2, 40.0, 68.9, 104.1, 108.2, 113.1, 114.0, 120.7, 121.4, 121.9, 122.9, 124.0, 136.9, 146.8, 148.8, 170.4, 200.4. Analysis for C₂₈H₂₇BrN₂O₃: Calculated C: 64.74, H: 5.24, N: 5.39; Found C: 64.49, H: 5.44, N: 5.38.

Benzyl 4-(6-bromo-1H-indole-3-yl)-2,6,6-trimethyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (Compound 23): Yield 81 %. m.p. 169-170 °C. ¹H-NMR (δ ppm, DMSO-d₆) 0.81 (3H; s; 6-CH₃), 0.95 (3H; s; 6-CH₃), 1.59-1.76 (2H; m; H-7), 2.27 (3H; s; 2-CH₃), 2.45-2.48 (2H; m; H-8), 3.67-3.69 (2H; dd; J: 6.8/2.0 Hz; OCH₂), 5.10 (1H; s; H-4), 6.83-7.52 (9H; aromatic protons), 9.16 (1H; s; HHQ N-H), 10.85 (1H; s; indole N-H). ¹³C-NMR (δ) 18.6, 18.8, 18.9, 24.1, 25.3, 34.0, 39.5, 40.1, 69.0, 103.0, 108.0, 112.9, 113.6, 120.8, 121.2, 121.5, 123.0, 124.2, 127.4, 127.9, 128.9, 129.0, 136.4, 137.1, 145.3, 149.0, 167.1, 199.4. Analysis for C₂₈H₂₇BrN₂O₃: Calculated C: 64.74, H: 5.24, N: 5.39; Found C: 64.46, H: 5.51, N: 5.40.

Benzyl 4-(6-bromo-1H-indole-3-yl)-2,7,7-trimethyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (Compound 24): Yield 76 %. m.p. 213-215 °C. ¹H-NMR (δ ppm, DMSO-d₆) 0.75 (3H; s; 7-CH₃), 0.99 (3H; s; 7-CH₃), 1.76 (1H; m; H-8a), 1.93-1.97 (1H; d; J: 16 Hz; H-8b), 2.12-1.16 (1H; d; J: 16 Hz; H-6a), 2.29 (3H; s; 2-CH₃), 2.49-2.50 (1H; m; H-6b), 3.33 (2H; s; OCH₂), 5.09 (1H; s; H-4), 6.92-7.74 (9H; aromatic protons), 9.18 (1H; s; HHQ N-H), 10.86 (1H; s; indole N-H). ¹³C-NMR (δ) 18.2, 18.9, 18.9, 26.4, 26.9, 27.2, 29.0, 32.0, 39.2, 40.1, 69.1, 103.6, 109.8, 110.7, 113.0, 121.5, 122.0, 122.6, 124.3, 127.5, 134.6, 144.1, 148.8, 167.0, 194.2. Analysis for C₂₈H₂₇BrN₂O₃ · 0.25 H₂O: Calculated C: 64.19, H: 5.29, N: 5.35; Found C: 64.18, H: 5.42, N: 5.41.

4.2. Instruments

All experiments were performed using a Gamry Reference 600 model (Gamry Instruments, PA, USA) potentiostat/galvanostat. A three-electrode system which formed of pencil graphite electrode (PGE) as working electrode, an Ag/AgCl electrode with saturated 3 M KCl as reference electrode and a platinum wire as counter electrode were used. The peak currents were measured automatically or manually. All measurements were performed at room temperature.

PGE a mechanical pencil as Model T 0.5 (Rotring, Germany) was used as a holder for pencil lead (Tombo, Japan). All leads have diameter of 0.5 mm and a total length of 60 mm. A metallic wire was wrapped around the metallic part of the pencil to supply electrical contact with the lead. 10 mm of lead was dipped in the working solution and this length corresponds to the 15.9 mm² active electrode area. Measurements were carried out by repeating on a new PGE surface using supporting electrolyte solution.

4.3. Procedures

4.3.1. Preparation of standard solution

A solution of all compounds and NIF (2000 mg/mL) was freshly prepared with supporting electrolyte. The studied compounds and NIF were used between 0.1 - 0.5 mg/mL of concentration range. Pure nitrogen gas was passed for sufficient time to remove oxygen from the solution.

4.3.2. Detection of antioxidant activities of 1,4- DHPs derivatives and NIF with DPV method

In this proposed study, PGE was dipped into 0.1 M TBAP-DCM supporting electrolyte and recorded

to obtain the original limiting current (I) of PGE electrode in the potential range of 0 and -2.00 V vs. Ag/AgCl reference electrode. Nitrogen gas was passed through the supporting electrolyte solution to remove oxygen and voltammogram was recorded and the residual peak current value (I_{res}) were obtained. 0.1 - 0.5 mg/mL concentration amounts of 1,4 DHPs or NIF compounds were added to the supporting electrolyte solution to obtain the decrease of the oxygen peak current. Voltammograms were scanned and electrochemical reduction current (I_{res}) were obtained corresponding to the concentration of the added antioxidant at constant potential.

The pulse amplitude (A), pulse increment (E_s), pulse time (W) and pulse period (T) parameters of DPV method were as follows: 50 mV, 20 mV, 0.1 s and 0.5 s, respectively were applied in this study.

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