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A combination risk assessment of paracetamol: electrochemical oxidation behavior and cytotoxicity evaluation effects of paracetamol in the presence of N^1,N^2-dibenzylethane-1,2-diamine

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Abstract

The cytotoxicity effect of paracetamol in the present of a diamine derivative was evaluated in the liver cell. In this study hydropyrazino-quinoxalinylidene-acetamide (HPQA) as agent toxicity of liver was synthesized in electrochemical cell as the simulated body environment by electrooxidation reaction. A direct electron transfer (DET) mechanism occurred during the process on the surface of the carbon anode. The electrochemical oxidation of paracetamol was studied using cyclic voltammetry and controlled-potential coulometry (CPC) techniques. The product was characterized by FT-IR, ^{1}H NMR, ^{13}C NMR and ESI-MS^{2} after purification. Cytotoxicity of final compound was evaluated using MTT assay on the *CCL-13* line cell of liver. The results indicate that the presence of amine derivatives lead to increased toxicity of paracetamol toxicity effects in the human body. Cell viability at concentration of 500µg/mL was 78% for paracetamol while viability of liver cells in present of product was 18% for 168 µg/mL. The cycloaddition mechanism was suggested according to the overall obtained results.

Key words: Cytotoxicity effect; Diamine; Electroorganic synthesis; Paracetamol

1. Introduction

Paracetamol is a valuable drug in widespread use in pharmaceutical industries. It is extensively used as analgesic and antipyretic drug. It is mainly used for the relief of mild to moderate pain associated with arthritis, head ache, back ache, and postoperative pain [1–3]. It is also used for treatment of fevers of bacterial or viral origin [4]. Electrochemical methods are widely used for the study of electro active compounds in pharmaceutical forms and physiological fluids due to their simple, rapid, and economical properties [5]. As an electro

active substance, paracetamol has also attracted much interest. Paracetamol is considered a safe drug at therapeutic level but in overdose states it produces renal necrosis in both humans and experimental animals [6]. In experimental animals, it causes hepatic depletion of glutathione at overdose level [7]. The first report was elucidated mechanisms of paracetamol-induced toxicity. It showed that a minor route of paracetamol metabolism involved its conversion by monooxygenases (cytochrome P450s) to a reactive arylating metabolite, known as *N*-acetyl-*p*-benzoquinone imine (NAPQI), which may cause acute tissue necrosis with toxic doses of this agent [8,9]. NAPQI causes a depletion of both the mitochondrial and cytosolic pools of reduced glutathione, thus cellular proteins are directly oxidized by the reactive metabolite [10]. NAPQI also inhibition of the antioxidant enzyme-glutathione peroxidase [11]. Because electrochemical oxidation very often parallels the cytochrome P450s catalyzed oxidation in liver microsomes, it was interesting to study the anodic oxidation of paracetamol in presence of a diamine derivative. A few studies have evaluated the effects of diamines on paracetamol. This encourages us to study electrochemical oxidation of paracetamol in the presence of N^1,N^2-dibenzylethane-1,2-diamine as nucleophile agent. The synthesized product was characterized by different techniques. Then, the *CCL-13* line was used as a model of liver progenitor cells to examine the cytotoxicity effects of paracetamol and the corresponding oxidized product (HPQA) in the presence of the diamine derivative. The best of our knowledge, this is the first report of electrochemical oxidation of paracetamol in the presence of diamine. Additionally, the electrochemical synthesis and the cytotoxicity effects of final product were simulated in the electrochemical cell.

2. Experimental

2.1. Chemical and electrochemical synthesis

2.1.1. Materials and methods

Reagent: The paracetamol (ACOP) was reagent grade material from Aldrich. The ammonium chloride and other acids and bases were of pro-analysis grade from E. Merck. Benzaldehyde, 99% and ethylenediamine were purchased from Aldrich. The reagents and solvents used in this study were of analytical grade and were used without further purification.

Apparatus: All electrodes for cyclic voltammetry experiments were from France Radiometer Analytical. Controlled-potential coulometry and cyclic voltammetry were performed using an Autolab model PGSTAT 302N potentiostat/galvanostat. The working electrode used in the voltammetry experiments was a glassy carbon disc (1.8 mm diameter). The glassy carbon was polished with polishing cloth before each measurement. A platinum wire was used as a counter electrode and the reference was a saturated calomel electrode (SCE). An undivided cell was used for controlled-potential coulometry [12]. The working electrode potentials vs. SCE were measured. The SCE reference and carbon rods were placed together, and their distance from the counter electrode was approximately 20 mm. A magnetic stirrer was used during electrolysis.

2.1.2. Chemical synthesis of N^1,N^2-dibenzylethane-1,2-diamine (3)

Benzaldehyde (4.24 g, 40 mmol) and ethylenediamine (1.20 g, 20 mmol) were mixed in an appropriate beaker in 100 mL MeOH. The obtained mixture was stirred overnight at room temperature, and then sodium borohydride (6.04 g, 160 mmol) was added. The mixture was refluxed for 2-3 hours, cooled and poured into 250 mL of H_2O. The solution was removed by filtration off and evaporated to dryness. The residue was then extracted with water-chloroform. The organic layer was separated and dried over anhydrous $CaCl_2$. The solvent was evaporated to yield yellow oil. The product (**3**) was characterized as a pure compound by FT-IR, ^{1}H NMR, ^{13}C NMR, and ESI-MS[2 13].

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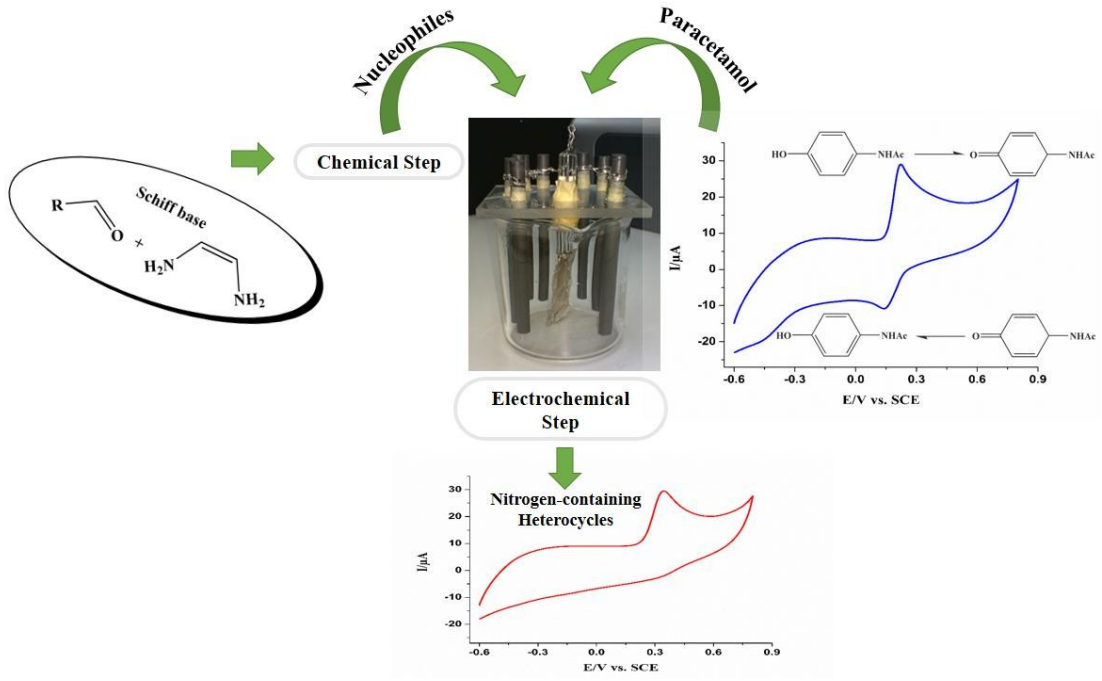
2.1.3. Electro-organic synthesis of final product (11)

For the electrodeposition experiments, in typical procedure, an aqueous solution of ammonia buffer (pH 9.0, 0.1mol/L) was pre-electrolyzed at 0.42 V versus SCE. Subsequently, 0.35 mmol of paracetamol (**1**) and 0.35 mmol of N^1,N^2-dibenzylethane-1,2-diamine (**3**) were added to the cell, and the electrolysis was performed using the same potential at room temperature (Scheme 1). To reactivate the graphite anode the process was cut off during the electrolysis and the anode washed in acetone. After completion the electrolysis, the precipitated solids that separated from the setup, were filtered, washed with distilled water and dried with sodium sulfate. The product was purified and characterized by several techniques including ^{13}C NMR, ESI-MS^2, ^{1}H NMR and FT-IR.



Scheme1 diagram of preparation process for the final product using electrooxidation reaction.

2.2. Characterization of products

2.2.1. N^1,N^2-dibenzylethane-1,2-diamine ($C_{16}H_{20}N_2$) (3)

Isolated yield = 67.6%, IR (KBr): 3304, 3084, 3061, 3027, 2924, 2829, 1950, 1877, 1811, 1603, 1584, 1494, 1452, 1407, 1358, 1202, 1108, 1074, 1054, 1028, 982, 911, 820, 736, 698

and 591 cm^{-1}. ^{1}H NMR, δ ppm (600 MHz $CDCl_3$): 2.01, 2.76, 3.77, 7.24-7.25, 7.27-7.31, 7.32-7.34. ^{13}C NMR, δ ppm (600 MHz $CDCl_3$): 48.6, 53.9, 126.9, 128.2, 128.45, 140.3. ESI-MS^2: *m/z*, calcd. 240.16; found 241.1 (M^++1)

2.2.2. *N*- (1,4,6,9-tetrabenzyl-10-oxo-1,2,3,4,6,7,8,9-octahydropyrazino [2,2 g] guinoxalin 5 (*10H*)-ylidene) acetamide ($C_{40}H_{39}N_5O_2$) (11)

Isolated yield = 94.3%, IR (KBr): 3027, 2924, 2376, 1528, 1296, 1240, 1161, 1074, 1028, 915, 827, 735 and 700 cm^{-1}. ^{1}H NMR, δ ppm (600 MHz $CDCl_3$): 2.121, 2.494, 2.497, 2.505, 2.511, 2.519, 2.521, 3.171, 3.179, 3.186, 3.200, 3.221, 3.559, 3.63, 3.785, 3.806, 3.850, 7.093, 7.105, 7.121, 7.133, 7.181, 7.195, 7.202, 7.209, 7.215, 7.227, 7.253, 7.260, 7.270, 7.285, 7.295, 7.39, 7.312, 7.318, 7.327, 7.337, 7.349 and 7.361.^{13}C NMR, δ ppm (600 MHz $CDCl_3$): 50.64, 56.92, 58.73, 76.84, 77.05, 77.26, 89.04, 126.61, 126.80, 127.00, 128.12, 128.24, 128.31, 128.59, 128.92, 129.13, 129.54, 139.19, 140.27. ESI-MS^2: *m/z*, calcd. 621.31; found 622.28 (M^++1).

2.3. Cytotoxicity assays

2.3.1. Reagents and apparatus of cytotoxicity assay

Reagent: Purified water, phosphate buffered saline solution (calcium and magnesium free), trypan blue (0.4%) solution, thiazolyl blue tetrazolium bromide (SIGMA), dimethyl sulfoxide (R&M Chemicals) and hydrogen peroxide (Univar). These chemicals were used without further purification.

Cell culture: American type culture collection Chang liver cell, (Homo sapiens, epithelial, *CCL-13*), with passage number 8.

Apparatus: Sterile plastic containers of issue culture grade: 75 cm^2 tissue culture flask (NUNC), 96-well tissue culture plate (NUNC) and 5 mL conical tubes (Falcon), haemocytometer counting chamber (Hirschmann Laborgerate), single and multichannel

pipettor (Eppendorf and Treff Lab), sterile 0.20 µm syringe filter unit (Sartorius), inverted microscope with phase contrast (Nikon Eclipse TS 100), carbon dioxide heat-coil-jacketed incubator (RS Biotech, model Galaxy R), biological safety cabinet class II (Bioair Instruments, model Aura 2000), microplate spectrophotometer (BIO-RAD, model xMark^{TM}).

2.3.2. Preparation of cell culture and exposure of test material

American type culture collection Chang liver cell was prepared for this study. Cells were grown as a monolayer at 37°C in a humidified atmosphere of 5% carbon dioxide and 95% air. The confluent monolayer was removed by trypsinisation and number of viable cells was calculated. Cells were seeded into a 96-well plate and incubated at 37°C for at least 12 hours or until attaining 80% confluency. The test material was tested at 15.63, 13.25, 62.5, 125, 250 and 500 µg/mL concentration. Growth medium from each well of a 96-well plate containing healthy culture was replaced with 200 µg of the test material solution. The cultures were then incubated for 24 hours at 37°C in a humidified atmosphere of 5% carbon dioxide and 96% air.

2.3.3. Cytotoxicity assays for paracetamol

In this study, the cytotoxic potential of the material paracetamol was assessed by determining the rate of cell proliferation. Cells were treated with varying concentration of test material and cytotoxicity was determined by assessing the rate of cell proliferation through the reduction of tetrazolium salts (MTT). Cell proliferation rate was obtained by dividing the mean optical density (OD) values of the test material with the mean OD of medium control.

2.3.4. Staining with MTT solution

5 mg/mL MTT solution was added into each well and incubated for 4 hours at 37°C in a humidified atmosphere of 5% carbon dioxide and 95% air. The optical density (OD) of test material and controls were determined by colorimetric method.

2.3.5. Statistically analysis

The rate of cell proliferation of test material is the percentage of cell viability calculated by dividing the mean optical density (OD) values of material with the mean OD absorbance of negative control, and multiplied by 100. Low cell viability value represents evidence of cytotoxic response.

2.3.6. Cytotoxicity assays of hydropyrazino-quinoxalinylidene-acetamide (HPQA)

The toxicity test of hydropyrazino-quinoxalinylidene-acetamide (HPQA) that synthesized from electrochemical step was carried out as a test material. Cells were treated with varying concentrations of the test material. Additionally, cytotoxicity was determined by assessing the rate of cell proliferation through the reduction of tetrazolium salts (MTT). Cell proliferation rate was obtained by dividing the mean optical density (OD) values of the test material with the mean OD of medium control.

3. Results and discussion

3.1. Chemical synthesis of N^1, N^2-dibenzylethane-1, 2-diamine (3)

As previously mentioned,[13] conventional method for the synthesis of Schiff bases is included reaction of aldehyde/ or ketone and amine in the presence of a catalyst under reflux conditions. In this work a simple method for the preparation of heterocyclic compounds based on Schiff base was provided without any catalyst in mild condition. This type of reaction condition can be suggested as a two-step mechanism. The first step is reversible, progressing through a carbinolamine intermediate and requires the removal of water by azeotropic distillation with benzene to achieve high yields. Although the reaction is acid catalyzed in the presence of amines, catalysts are not required generally. In the next step, the adduct containing two azomethine groups (C=N) reduced using sodium borohydride to provide of compound **3**. According to the results, the analytical and spectral data are completely consistent with the proposed formulation (supporting information).

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3.2. Electrochemical oxidation of paracetamol in the presence of N^1,N^2-dibenzylethane-1,2-diamine

The electrochemical behavior of paracetamol in the absence and presence of N^1,N^2-dibenzylethane-1,2-diamine has been investigated using cyclic voltammetry during the electrosynthesis on the electrode of a glassy carbon in an aqueous solution (pH 9.0) at room temperature.

Fig.. 1 (curve a) shows cyclic voltammetry of paracetamol **1**. This cyclic voltammogram shows one anodic peak (A_1) at +0.22 V .VS. SCE and the corresponding cathodic peak (C_1) at +0.148 V .VS. SCE, which correspond to the transformation of paracetamol **1** to *N*-(4-methylenecyclohexa-2,5-dienyl) acetamide **2** and *vice versa* within a quasi-reversible two-electron process. The oxidation of 1.0 mmol/L solution of paracetamol **1** in the presence of 1.0 mmol/L N^1,N^2-dibenzylethane-1,2-diamine **3** as a nucleophile in aqueous solution containing 0.1 M in the buffer solution NH_3/NH_4Cl (pH 9.0) was studied in some detail (Fig.. 1. Curve b). Under these conditions, the anodic peak (A_1) shifts to +0.34 V and the cathodic counterpart of it (peak C_1) disappears and the voltammogram shows an irreversible feature.

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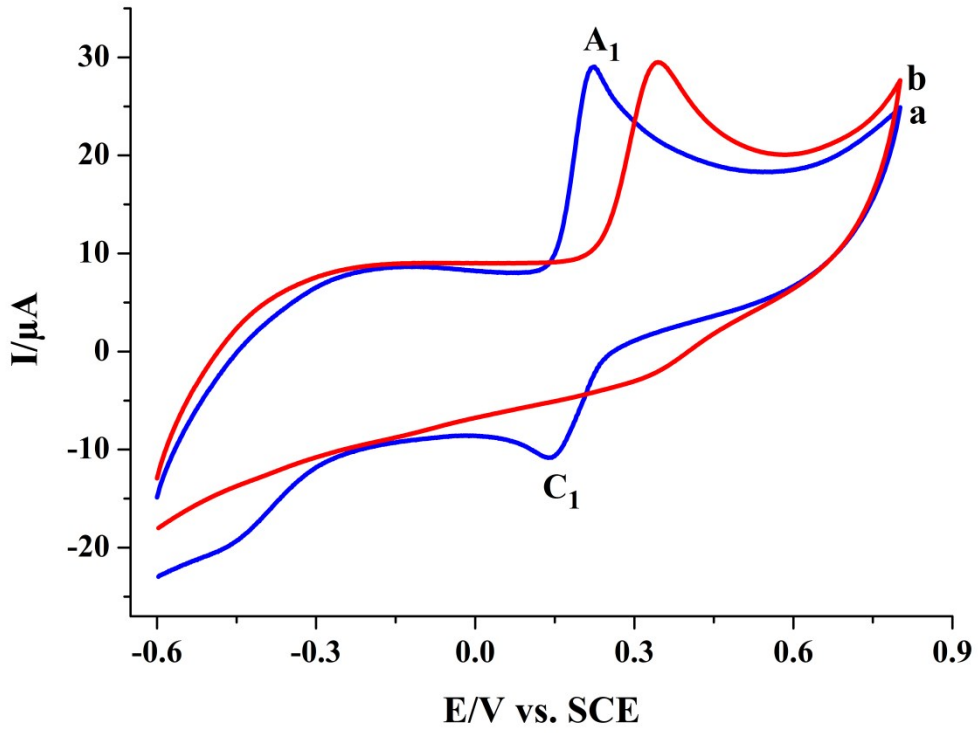

Fig. 1. Cyclic voltammograms of: (a) 1.0 mmol/L paracetamol; (b) 1.0 mmol/L paracetamol in the presence of 1.0 mmol/L N^1,N^2-dibenzylethane-1,2-diamine at a glassy carbon electrode (1.8 mm diameter) in the buffer solution NH_3/NH_4Cl (0.1 mol/L, pH 9). Scan rate: 50 mV/s; T=25 ± 1 ºC.

The effect of intensification of the potential sweep rate exhibits in Fig. 2 (curves a-g). It shows that the anodic peaks (A_1) have been increased by increasing of potential sweep rate while cathodic peak have been disappeared with increasing of potential sweep rate up to 250 mV/s. However, the cathodic peaks have slightly increasing at higher scan rates of 250-1000 mV/s (Fig. 2, inset). The variation of the peak current ratio (I_P^{C1} / I_P^{A1}) versus the scan rate for a mixture of paracetamol (**1**) and N^1,N^2-dibenzylethane-1,2-diamine (**3**) confirms the reactivity of **2** toward **3**.

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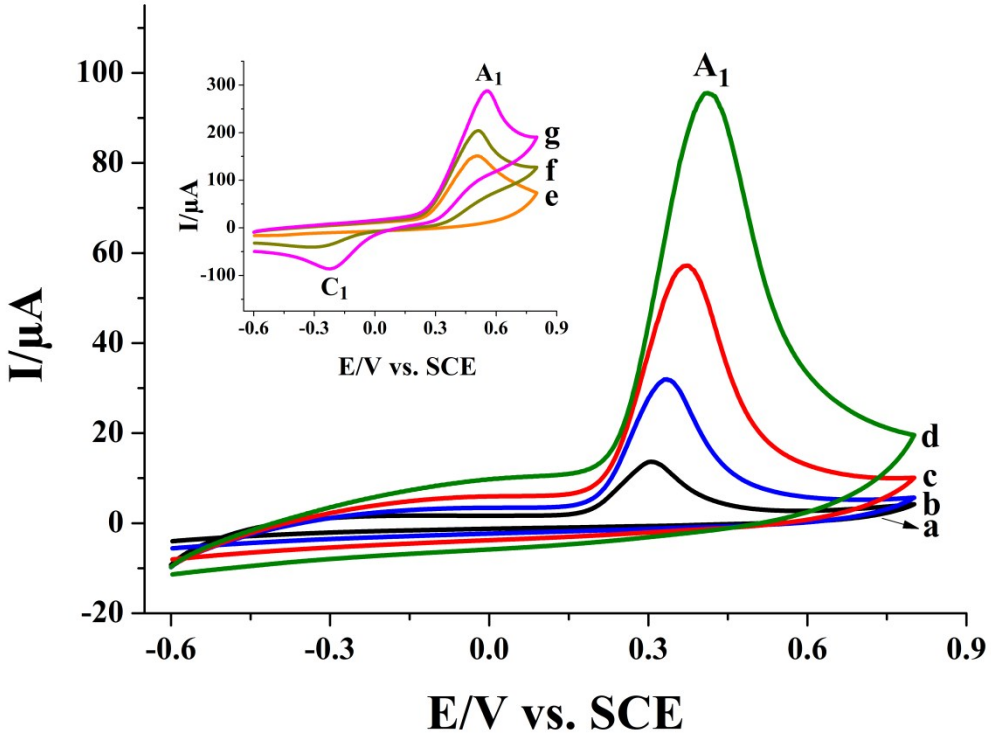

Fig. 2. Typical cyclic voltammograms of 1.0 mmol/L paracetamol in the presence of 1.0 mmol/L N^1,N^2-dibenzylethane-1,2-diamine in water containing of 0.1 mol/L NH_3/NH_4Cl as the buffer and supporting electrolyte (pH=9); at a glassy carbon electrode and at various scan rates. Scan rate from (a) to (d) are 10, 25, 50, and 100 mV/s, respectively. Inset: cyclic voltammograms of 1.0 mmol/L paracetamol in the presence of 1.0 mmol/L N^1,N^2-dibenzylethane-1,2-diamine at faster various scan rates. Scan rate from (e) to (g) are 250, 500 and 1000 mV/s, respectively. T=25 ± 1 ºC.

Fig. 3 shows the cyclic voltammograms of 1.0 mmol/L paracetamol in absent (curve a) and the presence (curve b) of 1.0 mmol/L N^1,N^2-dibenzylethane-1,2-diamine in acidic buffered solution pH 4. Results indicate that the height of cathodic peak do not disappears with decreasing of pH. This behavior is related to the low intermolecular Michael addition reaction rate of **2** toward **3** at acidic pHs. Because of the low reactivity at acidic pHs and the increase in the rate of Michael addition reaction between **3** and NAPQI (**2**) at alkaline pHs, the solution containing buffer NH_3/NH_4Cl (pH 9.0) was selected as the medium for a detailed electrochemical study.

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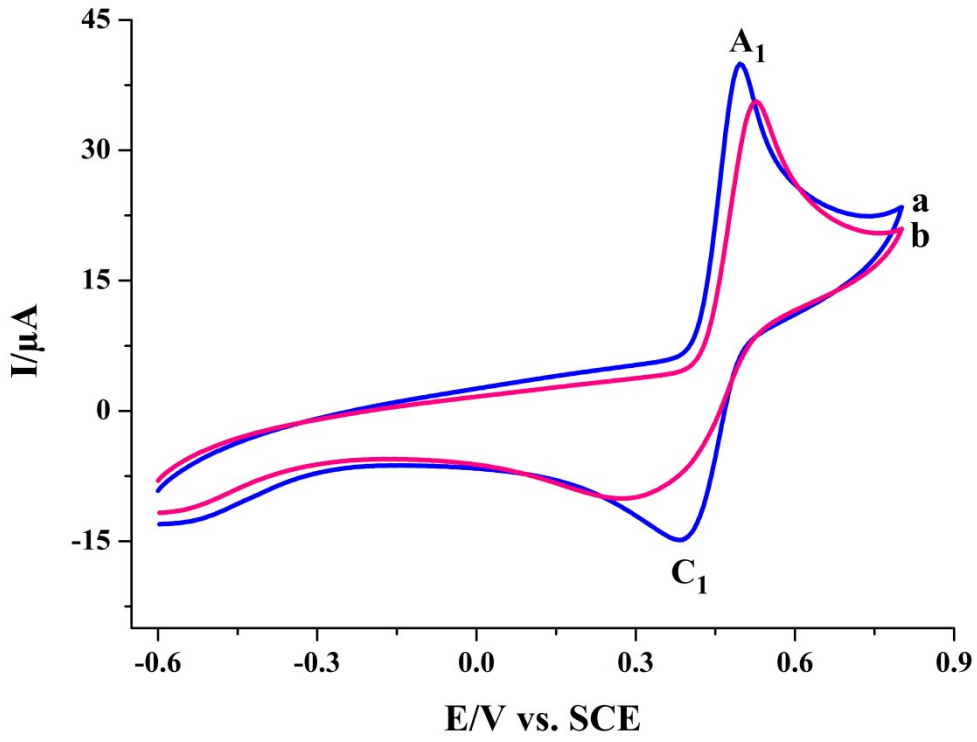

Fig. 3. Cyclic voltammograms of 1.0 mmol/L paracetamol in the presence of 1.0 mmol/L N^1,N^2-dibenzylethane-1,2-diamine in acidic buffered solution pH=4. Scan rate: 50 mV/s; T=25 ± 1 ºC.

To investigate the electrochemical properties of this reaction, controlled-potential coulometry was carried out in aqueous solution containing 0.35 mmol paracetamol and 0.35 mmol N^1,N^2-dibenzylethane-1,2-diamine in 0.1 M NH_3/NH_4Cl buffer solution during controlled-potential coulometry at 0.42 .V. VS. SCE. The electrolysis was monitored by cyclic voltammetry. As can be observed, Fig. 4 (curves a-e) shows the advancement of coulometry. Comparison of the current of peak A_1 (I_P^{A1}) shows a decrease in the current of peak A_1 during charge consumption linearly (Fig. 4, inset).

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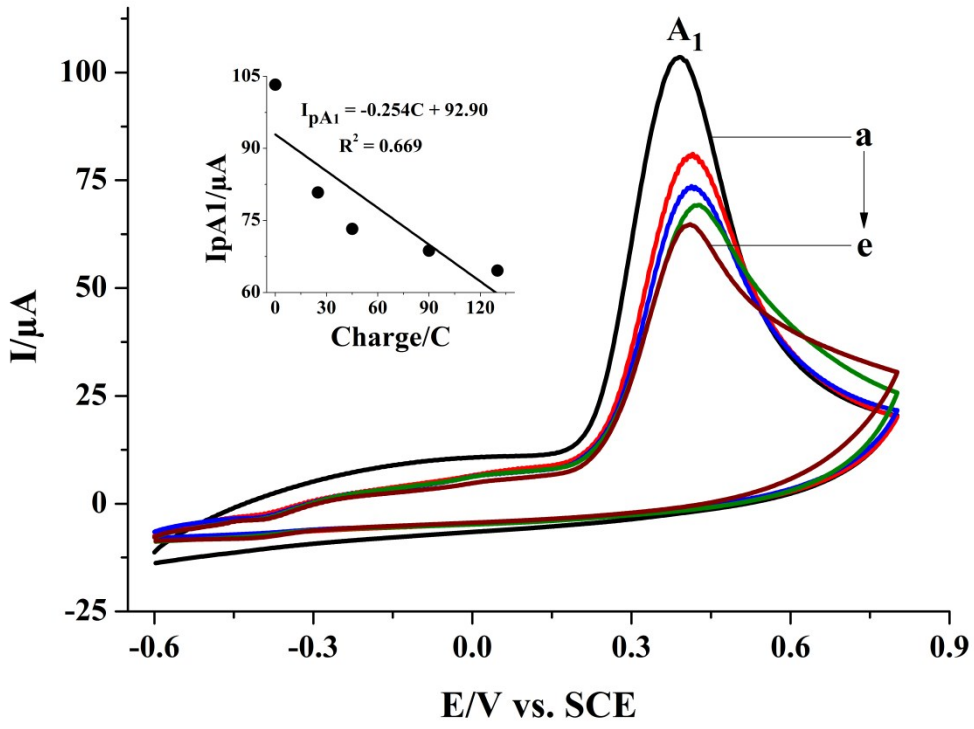

Fig. 4. Cyclic voltammograms of 0.35 mmol paracetamol in the presence of 0.35 mmol N^1,N^2-dibenzylethane-1,2-diamine in 0.1 mol/L NH_3/NH_4Cl buffer solution during controlled-potential coulometry at 0.42 V .VS. SCE. After consumption of: (a) 0, (b) 25, (c) 45, (d) 90 and (e) 130 C. Scan rate: 100 mV/s. T=25 ± 1 ºC. Inset: Variation of A_1 peak current versus consumed charge of 0.35 mmol paracetamol in the presence of 0.35 mmol N^1,N^2-dibenzylethane-1,2-diamine.

3.3. In vitro cytotoxicity

The results of MTT assay showed that there were no significant differences in viability of the cells incubated with paracetamol and control during the period of incubation when the concentrations of paracetamol were 15.63, 31.25, 62.5, 125, 250 and 500 µg/mL. Cell viability compared to the control for liver cells that incubated with 250 and 500 µg/mL of paracetamol was 84% and 78% respectively (Fig. 5 and Table 1). Paracetamol did not demonstrate a cytotoxic effect at concentration (500 µg/mL) under the condition of this test.

Table 1 Optical density values and Chang liver cell viability obtained after 24-hour exposure to the test material Paracetamol and controls.

	Negative Control	Positive Control	Paracetamol (µg/mL)					
			15.63	31.25	62.5	125	250	500
OD (550 nm)	1.449	0.400	1.332	1.537	1.612	1.214	1.258	1.190
	1.633	0.421	1.868	1.441	1.487	1.679	1.452	1.371
	1.619	0.403	1.318	1.711	1.382	1.464	1.259	1.141
Mean	1.567	0.408	1.506	1.563	1.494	1.452	1.323	1.234
SD	0.102	0.011	0.314	0.137	0.115	0.233	0.112	0.121
Viability (%)	100.00	23.59	96.11	99.74	95.32	92.68	84.43	78.75

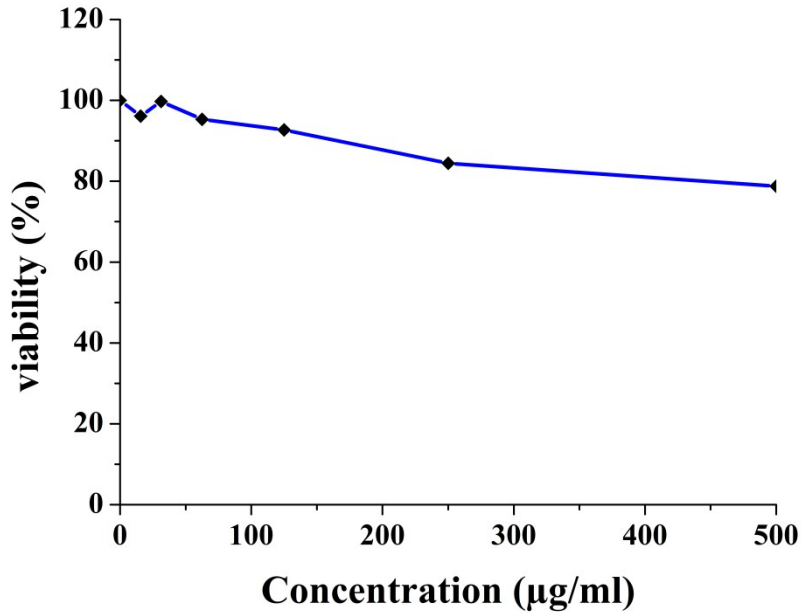

Fig. 5. Viability of Cheng liver cells at various concentration of the test material paracetamol.

Cytotoxicity of paracetamol in the presence of N^1,N^2-dibenzylethane-1,2-diamine was studied in the biological environment. The results show that there were considerable difference in viability of the liver cells incubate with hydropyrazino-quinoxalinylidene-acetamide (HPQA) and control during the period of incubation when the concentrations of HPQA were 42, 84, and 168 µg/mL (Table 2). The Fig. 6 shows the cell viability for liver cells with 168 µg/mL of HPQA were 18%. The test material hydropyrazino-quinoxalinylidene-acetamide demonstrated a cytotoxic effect at 168 µg/mL under the

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condition of this study. According to the results, concurrent use of paracetamol with amine derivatives causes toxicity of liver cells.

Table 2 Optical density values and Chang liver cell viability obtained after 24-hour exposure to the test material Aceta 1 and controls.

	Negative Control	Positive Control	HPQA (µg/mL)		
			42	84	168
OD (550 nm)	1.300	0.472	1.242	0.868	0.243
	1.388	0.517	1.137	0.897	0.245
	1.340	0.452	0.931	1.079	0.250
Mean	1.343	0.408	1.103	0.948	0.246
SD	0.044	0.033	0.158	0.114	0.004
Viability (%)	100.00	33.00	82	70	18

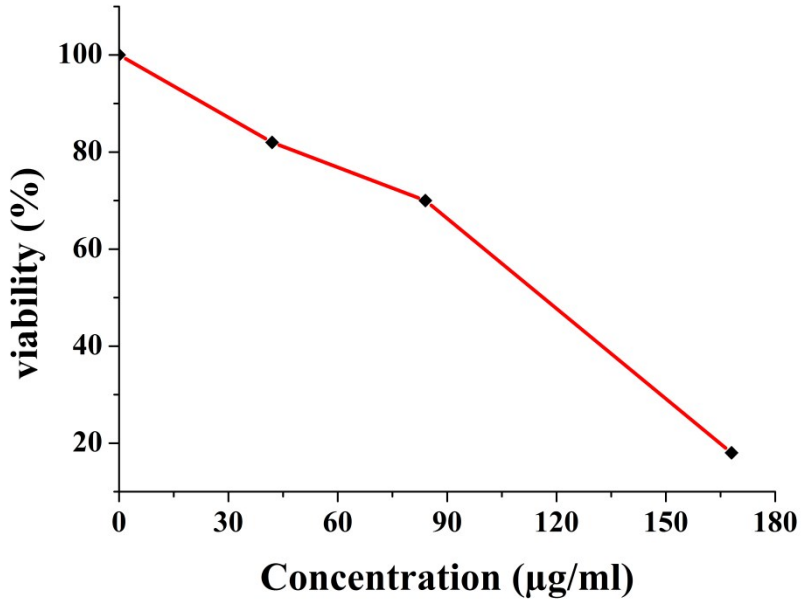
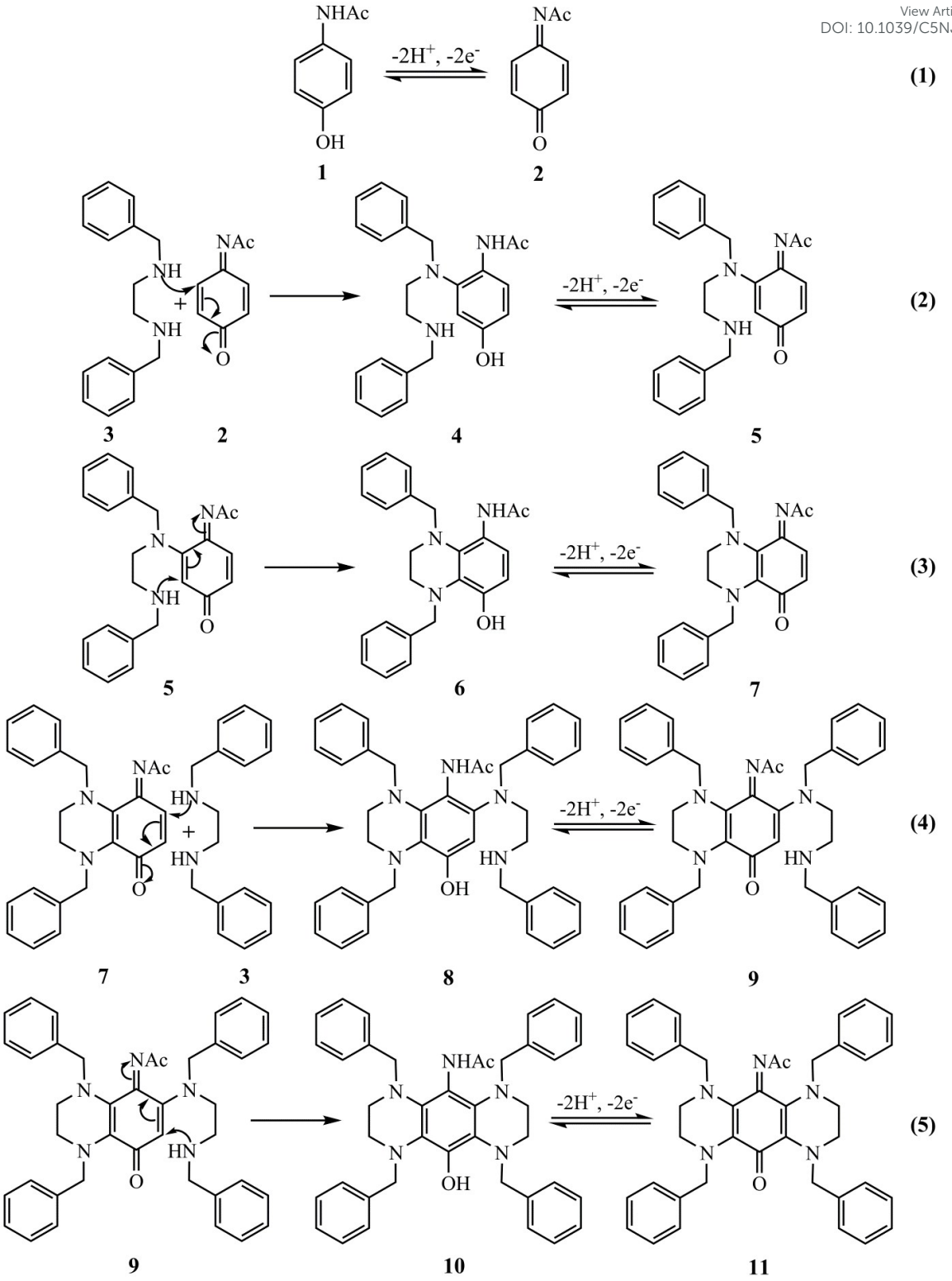


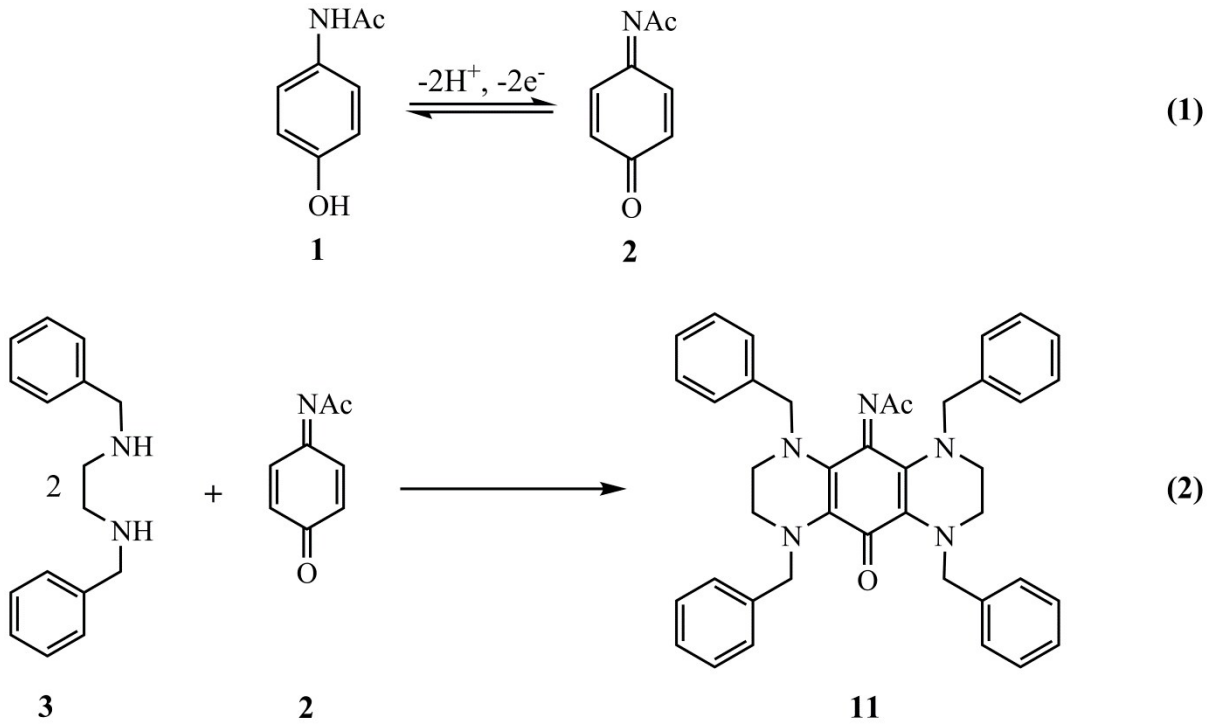
Fig. 6. Viability of Cheng liver cells at various concentration of the test material HPQA.

The proposed mechanism reaction of paracetamol in the presence of the mentioned amine derivative was suggested in Scheme 2.



Scheme 2 The proposed mechanism for the electrooxidation of paracetamol (**1**) in the presence of N^1,N^2-dibenzylethane-1,2-diamine (**3**).

Based on the obtained results, it seems that the anodic electrooxidation of paracetamol in the presence of diamine **3** follows the *ECECECECE* mechanism. Diagnostic criteria of cyclic voltammograms, coulometry, and spectroscopy allow us to propose the pathway illustrated in Scheme 3. According to Scheme 2, the diamine derivative **3** as nucleophile attacked to *N*-(4-methylenecyclohexa-2,5-dienyl) acetamide **2** and the intermolecular Michael addition reaction leads to intermediate **4**. This reaction is faster than other secondary electro reaction. Then, the secondary Michael addition reaction is occurred that leads to the formation of **5**. The final compound **11** was prepared by intermolecular Michael addition and cycloaddition reactions in the next steps. Moreover the oxidation take place through a 10 e^- solution electron transfer (SET) reaction.



Scheme 3 Overall proposed the pathway illustrated reaction of paracetamol in the presence of N^1,N^2-dibenzylethane-1,2-diamine.

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4.Conclusion

Since the aqueous media of electrochemical synthesis is similar to the biological environment, the toxicity of paracetamol in present of an amine derivative was evaluated on the liver cells by MTT assay in this study. The cytotoxicity measurement showed that the paracetamol has not significantly toxicity on liver cells and cell viability at concentration of 500µg/mL was 78%. While combination of paracetamol with a benzyl-ethylene-diamine derivative led to cytotoxicity effects on the liver cell. Based on the results, viability of this product was 18% for 168 µg/mL liver cells. The results indicate that existence of amine derivatives led to dramatically increasing of paracetamol toxicity effects in the human body. From the point of view of this method, the electro-synthesis method has some significant benefits. The use of electricity as energy instead of oxidative reagents, clean synthesis, one-step reaction, technical applicability, work in room temperature and pressure, and use of aqueous media instead of organic solvents, are of preeminent green advantages.

This study opens a new doorway to development of paracetamol electrochemical toxicity studies in the present of variety amine groups.

5. Supporting informations.

FT-IR spectra, ^{1}H-NMR spectrum, ^{13}C-NMR spectrum and ESI-MS^{2} spectrum of **3** and **11** products, also cytotoxicity test of paracetamol and HPQA are provided in supporting information.

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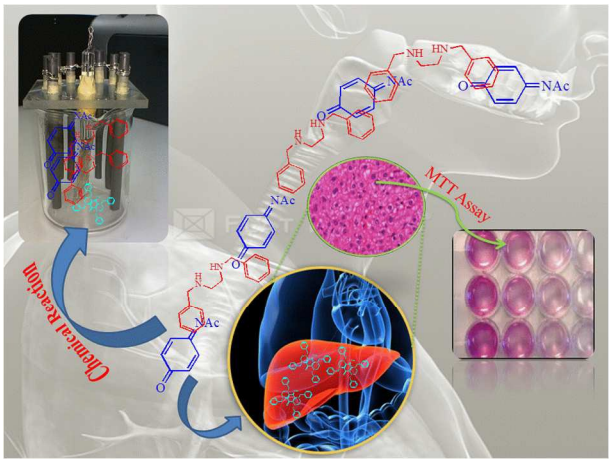

Simulation of paracetamol reaction in the presence of diamine in the liver using electrochemical cell