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Electrochemical Determination of Apigenin as An Anti-Gastric Cancer Drug in *Lobelia chinensis* Using Modified Screen-Printed Electrode

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We exploited a simple electrochemical method to activates bare screen printed carbon electrode and a novel amperometric apigenin sensor with high sensitivity was developed. Nickel nanoparticles (NiNPs) were doped on the activated screen printed carbon electrode (ACE), and then employed to investigate electrochemical behavior of apigenin in a 0.1 M B-R buffer solutions (50% ethanol, pH=3.0) by cyclic voltammetry (CV). Result shows a linear relationship of peak currents as a function of apigenin concentration in the range from 0.9 to 200 μM. Using proposed method, the apigenin in a herbal drug (Lobelia chinensis) can be easily determined in the absence of pre-separation. This simple and convenient method is of great potential application prospect for anti-gastric cancer drug analysis.

Keywords: *Lobelia chinensis*; Screen-printed electrode; Sensor; Ni nanoparticles; Anti-gastric cancer

1. INTRODUCTION

Helicobacter pylori, found usually in the stomach, was identified in 1982 for first time. It is linked to the development of peptic ulcer disease ulcers and gastric cancer. [1] Over 50% of the world's population is infected with Helicobacter pylori. Hence, it was classified as a group I carcinogen by WHO in 1994. Gastric adenocarcinomas can be divided into two categories. One is well differentiated (known as intestinal-type), that clinically manifests intestinal metaplasia and corpus-dominated gastritis with gastric atrophy. The other is undifferentiated (known as diffuse-type), that is characterized by gastritis throughout the stomach without atrophy.

According to the definition of Correa pathway, the development of intestinal-type adenocarcinoma is a multistep and multifactor process. Firstly, Helicobacter pylori infection induces chronic inflammation of gastric mucosa. Subsequently, chronic inflammation deteriorates to atrophic gastritis, intestinal metaplasia, dysplasia, and finally to adenocarcinoma. Appearance of atrophic gastritis is of crucial importance in the progress of gastric cancer development. Besides, high pH value in stomach, stimulators such as nitrates and salts will accelerate the exacerbation process. However, ascorbic acid and β-carotene can play the role of inhibitors. [2, 3]

Parietal, mucous neck, and chief cells are the major parts that are related to respond gastric juice, mucus, and enzyme secretions, respectively, in oxyntic gland of stomach [3]. Once Helicobacter pylori attaches to host cells, the immune system can respond immediately through cell ular signal transduction cascade. In the case of inflammation defense, extensive infiltration of monocytes and polymorphonuclear neutrophils into oxyntic glands will trigger a cytokine cascade and lead to dilation of gland. Simultaneously, mineralization of oxyntic parietal and chief cells occurs and futher progresses to focal fibrosis, then to complete loss (atrophic gastritis) [3-8].

Apigenin (5,7-Dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one), a belonging to the flavone class, is an important agent for the inhibition of Helicobacter pylori-induced gastric epithelial cell inflammation. As a natural product, it can be found in many plants, for instance vegetables and fruits, especially abundant in celery, onion, garlic, bell pepper, guava, passionflower, bilimbi fruit.[9, 10] Lobelia chinensis is a traditional Chinese medicine that contains high level of apigenin and other flavonoids (luteolin, and scopoletin). It widely distributes in East Asia countries including China, Japan, and Korea and has been used as a diuretic, antidote, and hemostat in traditional Chinese medicine. Although Lobelia chinensis is of great potential as a candidate agent for the inhibition of Helicobacter pylori, the analysis and research of apigenin in the Lobelia chinensis is challenging.

So far, various of techniques are utilized for analyzing flavonoid containing herbal medicine, for example high-performance liquid chromatography (HPLC) [11-16], Micellar electro kinetic capillary electrophoresis (MEKC), thin-layer chromatography (TLC) [17, 18], gas chromatography (GC) [19]. Nevertheless, analysis of apigenin using HPLC has many drawbacks, such as low resolution, long analysis time, and short column life time that caused by contamination. MEKC, TLC, GC methods often rely on photo absorption detector. Sensitivities of those method are relatively low. Moreover,heavy and costly relative instruments are requisite. Compared to above chromatographic methods, electrochemical methods like differential pulse voltammetry (DPV) is available to decrease the analysis time. Similar electrochemistry methods were developed successfully to study the mechanism of electrode [20-23]. Furthermore, investigation of redox properties of organic molecules using electrochemistry method can give insights into the metabolic fate, pharmacological activity and redox processes in vivo [24-27].

In the present work, a simple electrochemical method to activate bare screen printed carbon electrode was also introduced, and a novel amperometric apigenin sensor with high sensitivity was developed. The NiNPs doped ACE shows excellent properties for the detection of apigenin in a herbal drug (Lobelia chinensis). Interferences like ascorbic acid and metal ions with high concentration show tiny impact on the apigenin determination under the experimental conditions. This simple and convenient method shows potential application prospect for anti-gastric cancer drug analysis.

2. EXPERIMENTS

2.1. Chemicals

All purchased chemicals were without further purification: $Ni_2SO_4 \cdot 6H_2O$ (Sigma-Aldrich). Stock solutions (0.01 M) of apigenin were prepared by dissolving an accurate amount of apigenin (Checkout Institute of Biology Drugs) in 0.2 M sodium hydroxide solution, and then stored at 4 °C (protect from light), Screen printed carbon electrode were purchased from TianchengTeh Co., Ltd.

2.2. Electrochemical apparatus

Electrochemical experiments were conducted on a Model 650A electrochemical system (CHI Instrument Company, USA) employing a standard three-electrode electrochemical cell. A NiNPs doped ACE was used as working electrode; platinum (Pt) wire was used as auxiliary electrode; and a saturated calomel electrode (SCE) was used as reference electrode. pH were monitored by a Microprocessor pH Meter.

2.3. Fabrication of NiNPs modified ACE (NiNPs/ACE)

ACE preparations: firstly, a bare screen printed carbon electrode was washed in an ultrasonic bath containing ethanol-water mixed solution to clean the surface. Subsequently, the cleaned screen printed carbon electrode was transferred into an electrochemical cell containing PBS (pH=7) and KCl. Then, the potential was circulated from 0 to 2.0 V for 10 cycles to prepare activation of screen printed carbon electrode (ACE). After that, the obtained-ACE was dried at room temperature, and then transferred into the electrochemical cell containing Na_2SO_4 (0.1 M) and Ni_2SO_4 (0.02 M). Followed, the constant potential of -1.0 V was employed for 50s for the deposition of NiNPs on the ACE surface. Finally, the NiNPs doped ACE was obtained and used for the detection of apigenin without further treatment.

2.4 Electrochemical determination

In this work, a CHI 760 electrochemical workstation was employed. Besides, an Autolab electrochemical analyser (Eco Chemie, Netherlands) was used to perform the electrochemical impedance spectroscopy (EIS) under an alternating current voltage of 5.0 mV with a frequency ranging from 0.1 Hz to 10 kHz, where a mixture of $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1) with a concentration of 10 mM in the presence of KCl (0.1 M) was utilized as the supporting electrolyte. CV was conducted in Tris-HCl (pH 8.5) using three conventional electrode with 100 mV/s scan rate.

2.5. Real sample test

The Lobelia chinensis sample powder was prepared by grinding dried. 5.0 g of the sample powder was extracted with 100 mL ethanol for 30 min in an ultrasonic bath. After that, the solution was filtered into a 100 mL volumetric flask, and then diluted with distilled water. Obtained sample solution was stored in the dark environment. Before measurement, the sample solution was added into the electrolyte which was then transferred into a voltammetric cell for cyclic voltammetry measurements.

3. RESULTS AND DISCUSSION

Surface morphology of bare CE, ACE and NiNPs/ACE were studied by the scanning electron microscope (SEM), and images of different magnifications were illustrated in Fig.1. The SEM image of ACE exhibits a cracked surface indicating the formation of a large number of defects which is much more than that of bare CE. This result suggests that the electrochemical activation process greatly changed the surface properties of CE. Fig.1C shows the SEM image of NiNPs doped ACE. The presence of Ni on the ACE surface was confirmed by the EDX (Fig.1D). A clear Ni signal has been observed in the spectrum, indicating the ball like NPs formed at ACE is belong to Ni element. It can be seen from the figure 1C that the ball-like NiNPs were evenly dispersed on the surface of ACE. The average size of nickel nanoparticles is 49 nm.

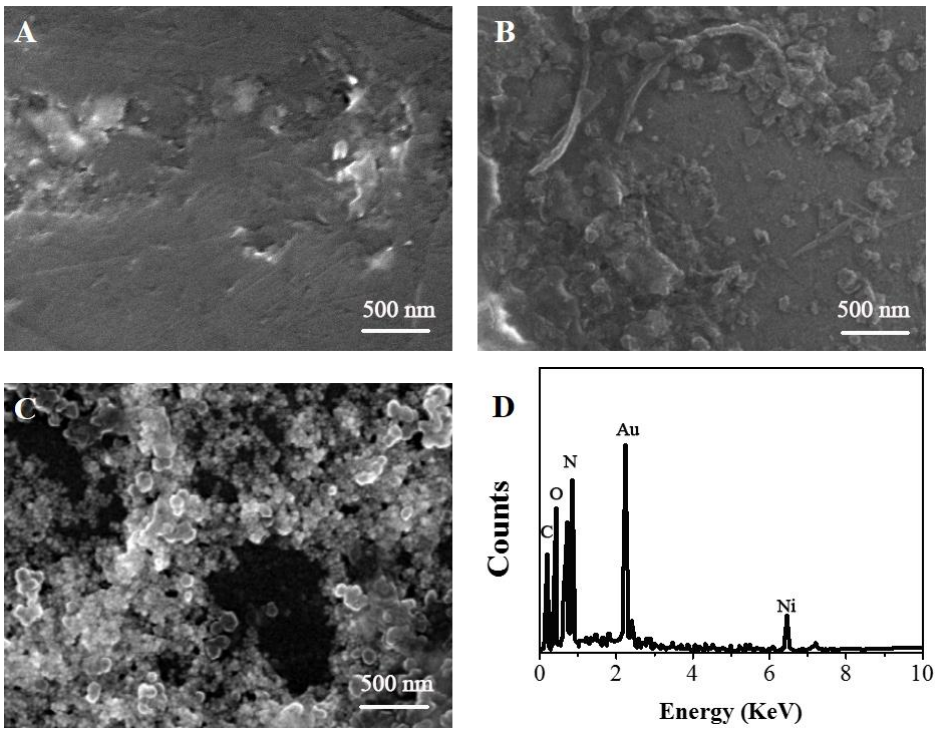

Figure 1. SEM images of CE (A), ACE (B) and NiNPs/ACE (C) and EDX result of NiNPs/ACE (D).

Impedance spectroscopy was recognized as an efficient approach to analyse the characteristics of the surface, which could allow to understand the chemical processes and transformation related to the surface of the conductive electrode. The impedance of bare CE, ACE and NiNPs/ACE between the electrode and electrolyte interfaces was carried on EIS, and the results were listed in Fig. 2. In Fig. 2A and Fig. 2B, the EIS spectra of bare CE, ACE and NiNPs/ACE in PBS containing $[Fe(CN)_6]^{3-/4-}$ (5 mM) and KCl (0.1M) and the Randles equivalent circuit model was demonstrated. The Rct, CdI and Zw represented as charge transfer resistance, double layer capacitance and Warburg impedance, respectively. Semicircle impedance was obtained from the parallel combination of Rct and CdI. It can be seen from the figure that the bare CE shows a large Rct vallue of 625Ω. In contrast, the Rct value of ACE and NiNPs doped ACE were only 247 Ω and 102 Ω, respectively. This indicates that the the electrochemical activation process and the doping of nickel nanoparticles on the surface of ACE both contributes to the decrease of charge transfer resistance. In addition, electron transfer kinetics of the NiNPs/ACE were also calculated from the impedance spectra. It can be clearly seen that the doping of NiNPs enhanced the electron transfer kinetics as well due to the outstanding electrochemical conductivity [28, 29]. Thus, the NiNPs/ACE shows the best electron transfer property.

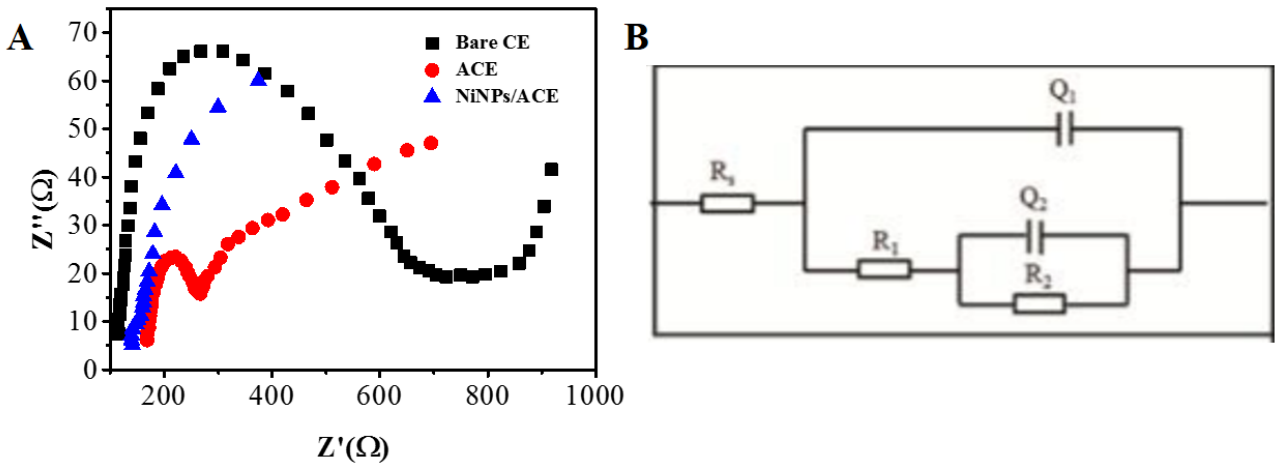

Figure 2. (A) EIS of bare CE , ACE and NiNPs/ACE in PBS containing $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1) with a concentration of 10 mM in the presence of KCl (0.1 M). (B) Randles equivalent circuit model used for EIS analysis.

The apigenin molecule has a planar structure that contains double bond and phenol moieties. Owing to those hydroxyl groups, apigenin shows electroactivity that can be detected by Cyclic voltammetry. Fig. 3(A) shows CV in the potential range of 0.5-1.45 V over different electrodes under reaction condition of 10 μM apigenin solution in 0.1 M B-R buffer solutions (50% ethanol, pH=3.0). In the figure, bare CE and ACE exhibited very week response to apigenin. However, for NiNPs/ACE, two oxidation peaks at potentials of 0.97 and 1.25 V were observed. It is worthwhile to notice that, except the oxidation reaction, reduction process has not been detected. This indicats that the oxidation reaction is irreversible. This may been considered as the reason why apigenin possesses antioxidant activity in vitro.

The voltammograms with multi-cycles over CE , ACE and NiNPs/ACE were also investigated with a scan rate of 0.05 V/s under 25 ^{o}C. The results were showed in Fig. 3(B). The peak current decreased with an increase of cycles. However, after the third cycle, the peak current value became stable. This may be due to the oxidized apigenin with poor solubility accumulating on the surface of the electrode during the electrochemical process. As a contrast test, the NiNPs/ACE was immersed into the apigenin solution for 10 min, and then it was transferred into 0.1 M $K_4[Fe(CN)_6]$ and buffer solution absence of apigenin, respectively, for CV performances. The CV results shows that tiny difference was observed when the apigenin immersed NiNPs/ACE was transferred into $K_4[Fe(CN)_6]$, and no oxidation peak can be found when NiNPs/ACE was in the buffer solution. Those results suggest that apigenin is not easy being adsorbed on the surface of NiNPs/ACE. However, when the NiNPs/ACE firstly performed a cyclic scan in the apigenin solution and then being transferred into 0.1M $K_4[Fe(CN)_6]$, no redox peak emerged. This reveals that oxidized apigenin can be strongly adsorbed on the surface of NiNPs/ACE. Therefore, in order to remove deposited oxidative production of apigenin from the electrode surface, a polish process and rinse with deionized water are indispensable.

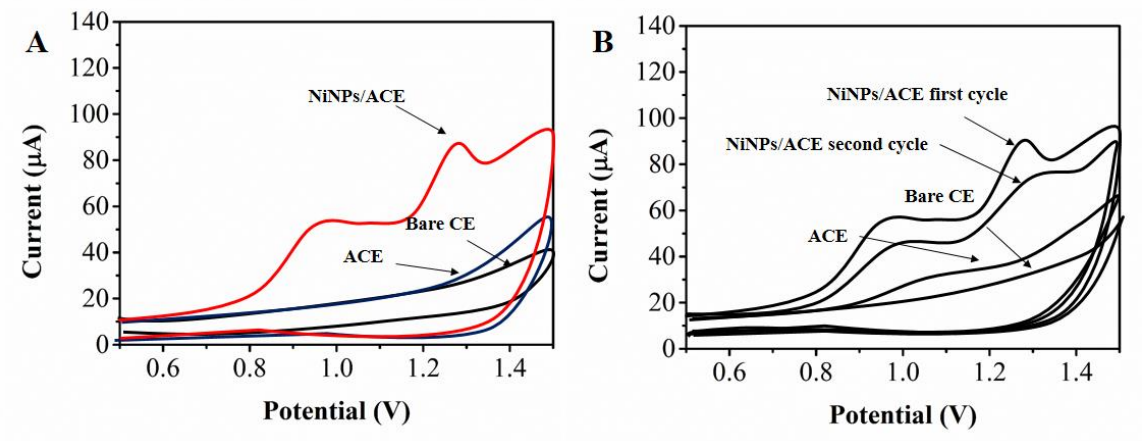

Figure 3. (A) The voltammograms of the CE , ACE and NiNPs/ACE under the condition of 10 μM apigenin. Condition: 0.1 M B-R buffer solution (50% ethanol, pH=3.0) (25 °C, scan rate 0.05 V/s); (B) The voltammograms with multi-cycles under the condition of 10 μM apigenin in 0.1 M B-R buffer solution (Same condition as Figure 3A).

Usually, if the electrode process is adsorption-controlled process, current presents as a linear function of scan rate. Differently, in the case of diffusion-controlled process, current presents as a linear function of square root of scan rate. Under the experimental conditions, when scan rate increased from 50 to 300 mV/s, the peak intensity and square root of scan rate presented a linear relation.

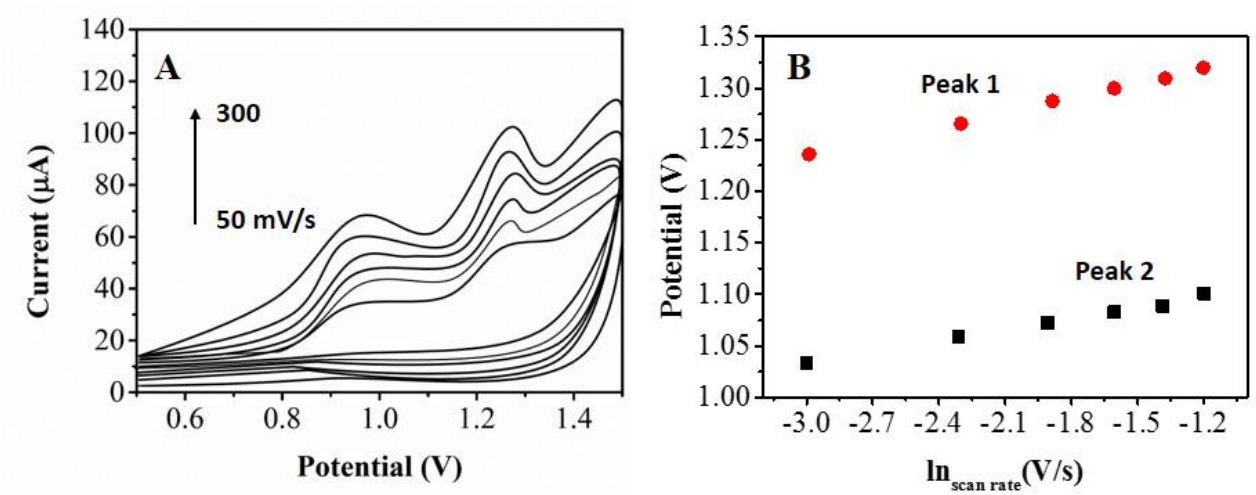

Figure 4. The cyclic voltammograms of apigenin under different scan rates (A). Relationship between the anodic peak potential (Ep) and the natural logarithm of the scan rate (*ln*v) for P1 and P2 (B). Condition: 0.1 M B-R buffer solution (50% ethanol, pH=3.0) (25 °C, scan rate 0.05 V/s)

This suggests that the electrode process of apigenin oxidation is diffusion-controlled. Fig. 4A shows the influence of scan rates on the CV peaks. From the figure, it can be seen that scan rate strongly affects the anodic peak potential, Ep, peak current, and Ip. Moreover, in Fig. 4B, Ep presented as a linear function of *ln*v under the potential scan rate from 50 to 300 mV/s. This result ensures the irreversible process of the electrochemical oxidation of apigenin. According to the Nicholson method, charge transfer coefficient of P1 were calculated to be 0.52, while P2 is 0.97. In addition, the electron participating in the electrode reaction process were also calculated. When α was assumed to be 0.5, the electron participating of P1 and P2 are $1e^-$ and $2e^-$, respectively. The influence of pH on the cyclic voltammetric response of apigenin was also examined between pH 2.0 and 10.0. The acidity of the solution was adjusted by H_2SO_4 or NaOH. The results revealed that the current value of ip for P1 and P2 decreases with an increasingvalue of pH, and stable responses and the best defined peak were obtained in the range of 3.0~4.0. At higher pH (for example, pH > 7.0), the anodic peak almost disappears. Based on the above discussion, a value of pH 3.0 was selected and the methods were developed at this pH value [30-32].

In this work, we used differential pulse voltammetry (DPV) for electrochemical measurements. As one of the most impactful electro analytical technique, differential DPV possesses well-established advantages, for instance, high sensitivity and low detection limits, good discrimination against background currents and precisely analyze of electrode reactions. Besides, due to a more stable response, P2 was utilized to analyze oxidation peak of apigenin. And based on the apigenin electrochemical oxidation process over NiNPs/ACE, an novel analysis method was developed to examine apigenin-containing samples as well. In Fig. 5, DPV recorded with an increasing amount of apigenin. As it can be seen that, with the increase of apigenin concentration in the range of 0.09 μM to 200 μM, a linear increase of peak currents was observed. Standard deviations (S.D.) of the slope and intercept were 0.00214 and 0.0177, respectively. Furthermore, the signal-to-noise ratio was calculated to be 3 which responds to the calculation of limit of detection (LOD). Therefore, the obtained sensitivity of our proposed method for the apigenin examination is 5 nM. The sensitivity of the

proposed sensor was compared with that of other reported modified electrodes and the results were presented in Table 1. This low detection limits might be attributed to the enormous loading of NiNPs which greatly amplified the stripping peak signals. In addtion, the repeatability of the proposed method was checked by measuring the oxidation process for ten times. Results shows that the R.S.D. of peak current of 5μM apigeninon over NiNPs/ACE was 4.7%, implying that proposed method is of good repeatability.

Table 1. Comparison of the present NiNPs/ACE with other apigenin determination methods.

Method	Linear detection range	Detection limit	Reference
HPLC	—	—	[33]
GCE	1.2-500μM	2μM	[31]
GCE	0.9-200μM	0.5μM	[30]
NiNPs/ACE	0.09-200μM	5 nM	This work

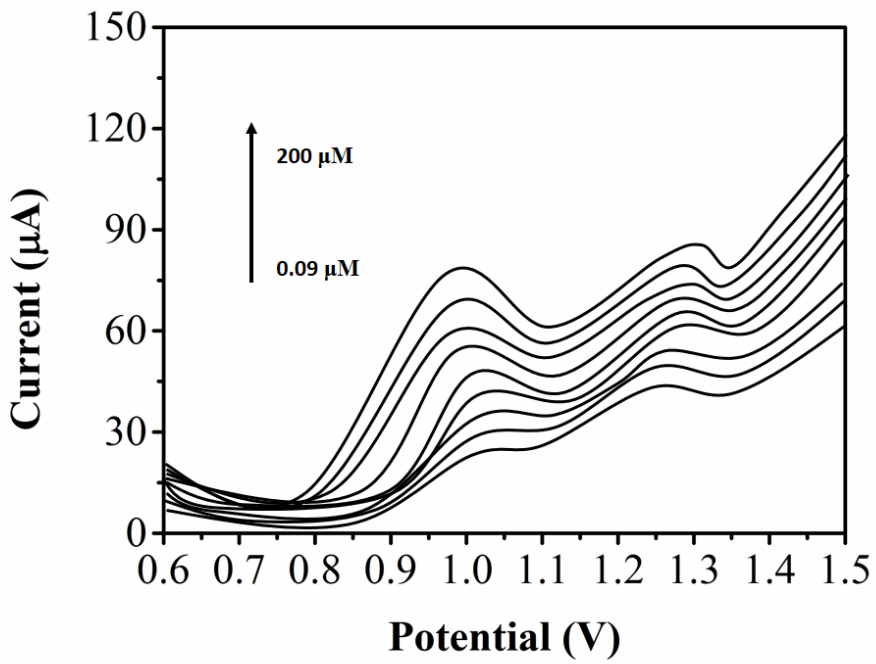

Figure 5. Typical differential pulse voltammograms of apigenin with different concentrations under test conditions. Condition: 0.1 M B-R buffer solution (50% ethanol, pH=3.0) (25 °C, scan rate 0.05 V/s)

Aforementioned method was utilized for analysis of apigenin.The influence of interfering substance on apigenin analysis in biological samples was also evaluated. Each sample contained a fixed amount of apigenin and excess amount of interfering substance. The tolerance limit is defined as the maximum concentration of the interfering substance, and error for determination of apigenin is less than ± 5% . Ascorbic acid, a representative potential interfering compound contained in biological

samples, was added in apigenin solution, and the influence of ascorbic acid on apigenin determination was investigated. It was found that no serious interference occurred under the experimental conditions. An equal or even higher molar concentration (25:1) of ascorbic acid to apigenin had no obvious impact on the apigenin analysis. Moreover, interferences of other metal ions such as Al(III), Cu(II), Fe(II), Cd(II), Pb(II), Zn(II) and Mg(II) were tested under the same experimental condition. The results show that no obvious influence on the apigenin determination under the condition of 200-fold excess of metal ions mixing with apigenin. On the basis of above conclusions, proposed method was successfully applied to analyze apigenin.

In addition, the proposed method above was utilized to detect apigenin in traditional Chinese medicine, for example Lobelia chinensis. Before the test, Lobelia chinensis was pretreated (see experimental section), and apigenin standard solution was added into the Lobelia chinensis solution (5.0 mL) for recovery detection. The obtained solution were parallel measured for six times and the results were listed in Table 2. The average contents and recoveries of apigenin detected in Lobelia chinensis solution are 0.0745 mg/g and 100.18%, respectively.

Table 2. The contents and recoveries of apigenin detected in Lobelia chinensis (n=3).

Sample	Added (mg/g)	Found (mg/g)	HPLC result (mg/g)	Recovery (%)
1	0.05	0.1241	0.1252	99.12
2	0.07	0.1455	0.1479	98.38
3	0.10	0.1721	0.1698	101.89
4	0.15	0.2235	0.2199	101.64
5	0.30	0.3801	0.3805	99.89

4. CONCLUSION

In the present work, a NiNPs/ACE was prepared using electro-deposition method and then applied to apigenin electrochemical determination. The as-prepared apigenin sensor shows a good linear analytical response in a wide apigenin concentration range from 0.09 μM to 200 μM. The limit of detection (LOD) of this sensor is 5 nM. Furthermore, apigenin sensor shows good selectivity of apigenin detection, even in the presence of interfering molecules. The proposed method can be also employed to detect apigenin in traditional Chinese medicine like Lobelia chinensis. This is of great potential to analyze clinic anti-gastric cancer drug.

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