Int. J. Electrochem. Sci., 13 **(2018)** 10193 – 10206, doi: 10.20964/2018.11.02

International Journal of
ELECTROCHEMICAL
SCIENCE
www.electrochemsci.org

Electrochemical Interaction Between Free Radicals and Lappaconitines

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Received: 3 April 2018 / *Accepted:* 2 July 2018 / *Published:* 1 October 2018

We used electrochemical analysis methods to study how the aconitum alkaloids lappaconitine and its salts (HBr-lappaconitine, HCl-lappaconitine) scavenge free radicals (superoxide anion radical, 2,2-diphenyl-l-picrylhydrazyl, hydroxyl radical and lipid peroxy radical) on a multiwall carbon nanotube-modified glassy carbon electrode. The experimental results showed that lappaconitine, HBr-lappaconitine and HCl-lappaconitine scavenge free radicals. Lappaconitine's free-radical scavenging capacity decreased in the order 2,2-diphenyl-l-picrylhydrazyl > hydroxyl radical > superoxide anion radical > lipid peroxy radical. HBr-lappaconitine's free-radical scavenging capacity decreased in the order 2,2-diphenyl-l-picrylhydrazyl > lipid peroxy radical > hydroxyl radical > superoxide anion radical. HCl-lappaconitine's free-radical scavenging capacity decreased in the order hydroxyl radical > 2,2-diphenyl-l-picrylhydrazyl > superoxide anion radical > lipid peroxy radical. Scavenging ability toward 2,2-diphenyl-l-picrylhydrazyl and lipid peroxy radical decreased in the order HBr-lappaconitine > lappaconitine > HCl-lappaconitine. Scavenging ability toward superoxide anion radical and hydroxyl radical decreased in the order lappaconitine > HBr-lappaconitine > HCl-lappaconitine. The results show that the lappaconitine and its salts can be developed as natural antioxidants because of their free-radical scavenging activities.

Keywords: Lappaconitine; Electrochemical characterization; Free radical; Antioxidant capacity

1. INTRODUCTION

Lappaconitine, as one kind of an important active alkaloid ingredient in Chinese herbal medicine, is prevalent in *Aconitum sinomontanum*. The root tubers of *Aconitum sinomontanum* are often used in Chinese medicine [1, 2]. The practical application indicates that the lappaconitine and HBr-lappaconitine exhibit clear anti-inflammatory, detumescence, antipyretic, local anaesthetic and

analgesic effects and that they cause no addiction and lead to few adverse reactions. Lappaconitines have potential antioxidant activity, which may be the main reason for the anti-inflammatory activity of *Aconitum sinomontanum*. Yet, no study of the antioxidant activity of the *Aconitum* alkaloid lappaconitine and its salts has yet been reported.

Moderate levels of free radicals have been shown to play an important role in cell differentiation, cell apoptosis, immune reactions and other biochemical events. Excessive levels of free radicals will, however, cause a series of biochemical reactions in organisms, such as disruption of molecular structures, tissue, cells, and subcellular compartments. This disruption will then cause functional damage, cardiovascular disease, cancer and caducity as the damage level gradually expands. Excessive levels of free radicals are the pathological basis of many diseases. Therefore, rational use of antioxidants can effectively prevent and treat certain diseases, promote anti-ageing and avoid enzyme inactivation, protein denaturation, DNA fragmentation, polysaccharide degradation, cell disintegration, damage to biofilm structures and death. Synthetic antioxidants often have certain toxic side effects, which has led to strict control of their use. By contrast, natural antioxidants have few side effects. Thus, the search for effective and inexpensive natural antioxidants with no or low toxicity has been an active investigative area, and the detections about free radicals are an essential part[3-10]. As one function of an electro-active material, the process of scavenging free radicals in vivo is similar to that on an electrode. Electron transfer is now acknowledged as an electrochemical property. Thus, electrochemical methods have a brilliant future in the study of natural antioxidants. In this work, we used Gansu aconite as a raw material to extract high-purity lappaconitines. By measuring their free-radical activity in vitro, we confirmed their ability to scavenge free radicals and quantified the antioxidant activity of *Aconitum* alkaloids.

2. EXPERIMENTAL

2.1. Materials

A CHI660 electrochemical workstation (Shanghai Chen Hua Instrument Co., Ltd.) was used in conjunction with a three-electrode system composed of a glassy carbon electrode (GCE) as the working electrode, a saturated calomel electrode (SCE) as the reference electrode, and a platinum wire as the auxiliary electrode. A UV lamp (Beijing Zhiyuan through Biotechnology Research Institute) was used for spectrophotometry. A PHS-3C precision acidity meter (Shanghai Instrument and Instrument Limited by Share Ltd.) was used for pH measurements. A biochemical incubator (Yangzhou Hongdu Electronics) and a refrigerator (Changling Limited by Share Ltd.) were used for temperature control at high and low temperatures, respectively. An ultrasonic system (Kunshan Ultrasonic Instruments) was used for mixing and dissolution. All measurements were carried out at 25 ± 1°C.

2.2. Reagents

Ascorbic acid and 2,2-diphenyl-l-picrylhydrazyl were from Sigma Chemical. Ovolecithin was from Laiyang Shandong Double Chemical Co., Ltd. Riboflavin and methionine were from Tianjin Tianxin Fine Chemical Development Center. Iron(III) chloride and green vitriol were from Tianjin BASF Chemical Co., Ltd. Hydrogen peroxide was from Tianjin Kaitong Chemical Co. Ltd. Multiwalled carbon nanotubes were from Sun Nanotech Co., Ltd. and were activated before experiments.

Britton-Robinson buffer solution was a mixed liquor with H_3BO_3, H_3PO_4, CH_3COOH and 0.2 mol/L NaOH in special proportions. The B-R buffer solutions were adjusted to pH 2.0 to 12.0.

2.3. Pretreatment of electrode

The glassy carbon electrode was polished with abrasive paper for metallography. It was then polished with a mixture of 0.05 μm Al_2O_3 powder and water. The glassy carbon electrode was washed with distilled water and dried at room temperature.

2.4. Activation of MWNTs

MWNTs (0.5 g) were placed in 100 mL of 6 M HCl and ultrasonicated for 4 h. The MWNTs were then washed with deionized water until the pH was neutral. The purified MWNTs were dispersed in 100 mL of mixed acid liquor (V_{HNO3}:V_{H2SO4} = 1:3) with ultrasonication for 6 h and then washed until neutral with deionized water. The MWNTs were then considered functional. The functional MWNTs were placed in an oven (100°C) for 12 h for drying. The multiwalled carbon nanotubes were characterized by transmission electron microscopy (TEM) and infrared spectroscopy.

2.5. Preparation of MWNT dispersion

MWNTs (5 mg) were added to 5 mL of distilled water and then ultrasonicated for 1 h to make a 1 mg/mL dispersion.

2.6. Preparation of modified electrode

A 10 μL dispersion of MWNTs was placed onto a treated glassy carbon electrode. They were dried in an oven (40°C) to produce a MWNT/GCE.

2.7. Preparation of lappaconitines and free-radical reaction systems

The lappaconitine was firstly extracted and purified freshly from root tubers of *Aconitum sinomontanum*. Then the purified lappaconitine was dissolved and recrystallized in hydrobromic acid

(HBr) and hydrochloric [chlorhydric] acid (HCl), respectively. The recrystallization processes were repeated three times. Finally, the HBr-lappaconitine and HCl-lappaconitine were obtained, respectively.

Preparation of hydroxyl free-radical reaction system: Green vitriol (0.1510 g) was accurately weighed into a 50 mL beaker. Thirty millilitres of double-distilled water was added, and the solution was sonicated for 10 min. After the sample was dissolved, the solution was diluted to 50 mL and labelled solution A. Hydrogen peroxide solution (0.0564 g) was accurately weighed into a 50 mL volumetric flask and diluted to volume with double-distilled water; the resultant solution was labelled solution B. All solutions were stored at 4°C for further experiments.

Preparation of superoxide free-radical reaction system: Lactochrome (0.0101 g) was diluted to 10.00 mL with 70% methanol, and the resultant solution was labelled solution C. Methionine (0.0102 g) was diluted to 10.00 mL with methanol, and the resultant solution was labelled solution D.

Preparation of 2,2-diphenyl-l-picrylhydrazyl free-radical reaction system: 2,2-Diphenyl-l-picrylhydrazyl (0.0200 g) was accurately weighed and diluted to 50.00 mL with methanol; the resultant solution was labelled solution E.

Preparation of lipid peroxide free-radical reaction system: Lecithin (0.5020 g) was dissolved in 50 mL of phosphate buffer (50 mmol/L, pH = 7.4) and treated with ultrasound in an ice-water bath for 2 h; the resultant solution was labelled F. Iron (III) chloride (0.0151 g) was diluted to 50.00 mL with methanol, and the resultant solution was labelled G. Ascorbic acid (0.0105 g) was diluted to 50.00 mL with methanol, and the resultant solution was labelled H.

2.8. Free-radical scavenging test method

Scavenging activity of hydroxyl radical. Experimental group: Solution X(Y,Z) (5.0 mL), solution A (2.0 mL) and the solution B (2.0 mL) were mixed and incubated at 37°C in the dark for 2 h. Control group: Solution X(Y,Z) (5.0 mL), solution A (2.0 mL), and double-distilled water (2.0 mL) were mixed and incubated at 37°C in the dark for 2 h.

Scavenging activity of superoxide radical. Experimental group: Solution X(Y,Z) (2.0 mL), solution C (1.0 mL), and solution D (1.0 mL) were mixed and incubated under an ultraviolet lamp at 254 and 365 nm in a dark location for 2 h. Control group: Solution X(Y,Z) (2.0 mL) and methanol (2.0 mL) were mixed and incubated under an ultraviolet lamp at 254 and 365 nm in a dark location for 2 h.

Scavenging activity of 2,2-diphenyl-l-picrylhydrazyl radical. Experimental group: Solution X(Y,Z) (2.0 mL) and solution E (2.0 mL) were mixed and incubated at 37°C in the dark for 2 h. Control group: Solution X(Y,Z) (2.0 mL) and methanol (2.0 mL) were mixed and incubated at 37°C in the dark for 2 h.

Scavenging activities of lipid peroxyl radical. Experimental group: Solution X(Y,Z) (1.0 mL), solution F (5.0 mL), solution G (2.0 mL), and solution H (1.0 mL) were mixed and incubated at 37°C in the dark for 4 h. Control group: Solution X(Y,Z) (1.0 mL), phosphate buffer (5.0 mL), solution G (2.0 mL), and solution H (1.0 mL) were mixed and incubated at 37°C in the dark for 4 h.

2.9. Calculation of free-radical scavenging rate

In the free-radical scavenging experiment, the solution contents changed after free-radical reaction with anti-free radicals. This change is reflected in the cyclic voltammetry curve as a corresponding change in the peak current. In general, the concentration of free radicals can be reduced or the free radicals can disappear and the corresponding peak current will change. When the component content of the free-radical scavenging effect is basically unchanged, the corresponding peak current remains the same. For a free-radical experimental system, through the experimental group and control group for changes in the peak current, the following formula can be used to evaluate the free-radical scavenging rate of the corresponding components:

$$\text{Radical scavenging}(\%)=\frac{\text{Ip(control)}-\text{Ip(exp)}}{\text{Ip(control)}}\times 100\%$$

where Ip (control) is the peak current of the control group and Ip (exp) is the peak current of the experimental group.

2.10. Experimental method

Fifteen millilitres of B-R buffer solution was placed in a sealed electrolytic cell. The three-electrode system was treated with 20 μL to conduct cyclic voltammetric scanning in the range of the potential. A clear oxidation peak appeared. The possible electrochemical processes (oxidation and reduction) are shown in Figure 1, and may be due to pores and channels on the surface of the modified multi walled carbon nanotubes modified carbon paste electrode. Molecules and ions may diffuse into these microporous layers. The microporous layers greatly increased the electrode specific surface area and the resulting currents in the voltammetric measurements. The MWNT-modified carbon paste electrode surface provided a favourable microenvironment for the electrochemical determination of alkaloids. The enhancement suggested that the MWNT-modified carbon paste electrode catalysed the electrochemical reaction of alkaloids because of a higher electron transfer rate, higher activity, and a larger specific surface area. All these special physical and chemical properties enhance the electron transfer on the modified glassy carbon electrode and greatly improve the response signal.

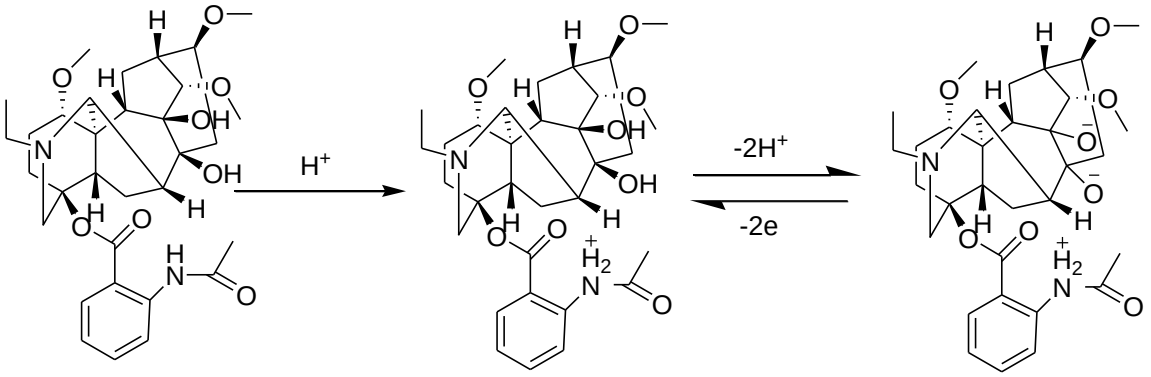

Figure 1. The possible reaction process of lappaconitine on the electrode.

3. RESULTS AND DISCUSSION

3.1. Selection of experimental conditions and electrochemical interaction between free radicals (hydroxyl radical, superoxide anion radical, 2, 2-Diphenyl-l-picrylhydrazyl, lipid peroxyl radical) and lappaconitines (lappaconitine, HBr-lappaconitine and HCl-lappaconitine)

The research about electrochemistry characterization of molecules with different structures has been carried out in various areas[11-35]. The researches for the preparation of HBr-lappaconitine were carried out by T. G. Tolstikova [36, 37], however, the detection of electrochemical interaction between free radicals and lappaconitines is rare. The experiment conditions between free radicals and lappaconitines need selection and optimization. In the B-R buffer solution at pH 5.8, the oxidation peak currents of lappaconitines were the maximum of the determinants; therefore, we chose the Britton-Robinson buffer solution as a testing medium. To improve the sensitivity of testing, instrumental parameters, including the initial potential (Init E, 1.0 V), final potential (Final E, -1.0 V), high potential (High E, 1.0 V), low potential (Low E, 1.0 V), quiet time, 2.0 s, and the scan rate 50.0 mV/s, were optimized appropriately [38, 39]. The test results show that the peak current of lappaconitines increased dramatically.

The lappaconitines in the bioactive components of root tubers of *Aconitum sinomontanum* for scavenging hydroxyl radicals' capacity were examined. In the reaction system[3] the hydroxyl radicals were produced and then attack the lappaconitines. The results for the root tubers of *Aconitum sinomontanum* extract with or without hydroxyl radicals treatment are shown in Figure 2 a and b (lappaconitine), Figure 3 a and b (HBr-lappaconitine), and Figure 4 a and b (HCl-lappaconitine). The superiority of the electrochemical analysis combined with free radicals screening method was obvious in the detection of any changes of the electrochemical interaction between free radicals and lappaconitines, which can be reflected by the peak current, although in the presence of interference components. The reaction between lappaconitines and hydroxyl radicals resulted in the transfers of one or more atoms from the lappaconitines to the free radicals occurred. Finally, the molecular structure would be destroyed. The peak signals of the lappaconitines would decrease or disappear in the cyclic voltammograms. For the molecules without free radical-scavenging capacity, there was no change in their cyclic voltammograms after reaction with free radicals.

The cyclic voltammograms of lappaconitine, HBr-lappaconitine and HCl-lappaconitine scavenging hydroxyl radical are shown in Figures 2, 3, and 4, respectively. The cyclic voltammograms were recorded with the control and experimental samples in pH 5.8 B-R buffer. An oxidation peak appeared between -0.2 and 0.0 V in the cyclic voltammogram, and a larger current was observed for the control sample. Compared with the control sample, however, a weaker current was observed from the experimental sample, likely because of the hydroxyl radical scavenging of lappaconitine, HBr-lappaconitine and HCl-lappaconitine.

The results for the root tubers of *Aconitum sinomontanum* extract with or without hydroxyl radicals treatment (HBr-lappaconitine) are shown in Figure 3 a and b. The results for the root tubers of *Aconitum sinomontanum* extract with or without hydroxyl radicals treatment (HCl-lappaconitine) are shown in Figure 4 a and b. The scavenging capacity of lappaconitine, HBr-lappaconitine, and HCl-lappaconitine with hydroxyl radical were calculated and compared, the results are shown in Table 1.

Based on the same experimental idea described above, we further performed other free radicals (including superoxide anion radical, 2, 2-Diphenyl-l-picrylhydrazyl, and lipid peroxyl radical) screening experiments on lappaconitine, HBr-lappaconitine and HCl-lappaconitine.

The cyclic voltammograms of lappaconitine, HBr-lappaconitine and HCl-lappaconitine scavenging the superoxide anion radicals are shown in Figures 5, 6 and 7, respectively. In Figures 5, 6 and 7, curve a represents the experimental cyclic voltammetry group (after reactions), and curve b represents the control group of cyclic voltammetry (before reaction). The graphs show an oxidation peak between -0.2 and 0 V, and the oxidation peak current of the experimental group was less than that of the control group. The results show that the lappaconitine, HBr-lappaconitine and HCl-lappaconitine substantially inhibit the production of superoxide anion; however, some differences were observed among the results.

The cyclic voltammograms of lappaconitine, HBr-lappaconitine and HCl-lappaconitine scavenging 2, 2-diphenyl-l-picrylhydrazyl are shown in Figures 8, 9 and 10, respectively. An oxidation peak appeared between -0.2 and 0 V, and the oxidation peak current of the experimental group was less than that of the control group. Therefore, the 2, 2-diphenyl-l-picrylhydrazyl radical scavenging effects of lappaconitine, HBr-lappaconitine and HCl-lappaconitine were observed; however, some of the differences in the results may be due to the introduction of HCl or HBr.

The cyclic voltammograms of lappaconitine, HBr-lappaconitine and HCl-lappaconitine scavenging lipid peroxyl radicals are shown in Figures 11, 12 and 13, respectively. The results show that in the lipid peroxyl radicals scavenging, lappaconitine and HBr-lappaconitine were oxidatively degraded in the free-radical reactions, which led to a decrease in the peak current. Thus, the oxidation peak current of the experimental group was less than that of the control group. The HCl-lappaconitine showed weak lipid peroxyl radical scavenging capacity.

3.2. Scavenging capacity of lappaconitine, HBr-lappaconitine and HCl-lappaconitine on different free radicals

The scavenging capacity of lappaconitine and HBr-lappaconitine and HCl-lappaconitine in these radical systems was investigated [3-5]. The results show that lappaconitine, HBr-lappaconitine and HCl-lappaconitine have certain effects on free radicals (Table 1). Lappaconitine and HBr-lappaconitine had the highest removal rates on 2, 2-diphenyl-l-picrylhydrazyl, respectively, 51.6% and 60.7%. HCl-lappaconitine exerted the highest scavenging rate on the hydroxyl radical, which was 26.9%. For the free-radical scavenging ability of the three substances, lappaconitine and HBr-lappaconitine exhibited stronger abilities than the HCl-lappaconitine. This result is possibly explained by the HBr-lappaconitine's $\bullet Br^-$ transferring electrons to •Br, which is relatively stable and can exist longer. Thus, the scavenging effect on free radicals is clear. By contrast, the HCl-lappaconitine's $\bullet Cl^-$ loses electrons and forms •Cl, which is not very stable compared with •Br [40-42]. Therefore, the scavenging effect of free radical was not clear in HBr-lappaconitine. In the case of the 2, 2-diphenyl-l-picrylhydrazyl and lipid peroxy radicals, the scavenging ability of HBr-lappaconitine was better than that of lappaconitine. For the superoxide anion radical and hydroxyl radical, the scavenging ability of lappaconitine was stronger than that of HBr-lappaconitine.

Table 1. Scavenging capacity of lappaconitines with four free-radical systems (n = 3)

Free radicals	Lappaconitine			HBr-lappaconitine			HCl-lappaconitine		
	Peak current (control) /μA	Peak current (exp) /μA	Scavenging capacity/%	Peak current (control) /μA	Peak current (exp) /μA	Scavenging capacity/%	Peak current (control) /μA	Peak current (exp) /μA	Scavenging capacity/%
Hydroxyl radical	-2.942 ±0.062	-1.535 ±0.046	47.8%	-2.833 ±0.030	-1.756 ±0.322	38.0%	-2.468 ±0.205	-1.804 ±0.080	26.9%
Superoxide anion radical	-4.928 ±0.371	-3.344 ±0.082	32.1%	-2.363 ±0.234	-1.841 ±0.132	21.8%	-3.808 ±0.103	-3.009 ±0.092	20.1%
2,2-Diphenyl-l-picrylhydrazyl	-2.664 ±0.029	-1.281 ±0.039	51.6%	-3.803 ±0.197	-1.496 ±0.056	60.7%	-3.476 ±0.342	-2.762 ±0.113	20.5%
Lipid peroxy radical	-4.029 ±0.358	-2.895 ±0.089	28.1%	-5.129 ±0.562	-2.185 ±0.245	57.4%	-4.793 ±0.186	-3.922 ±0.014	18.2%

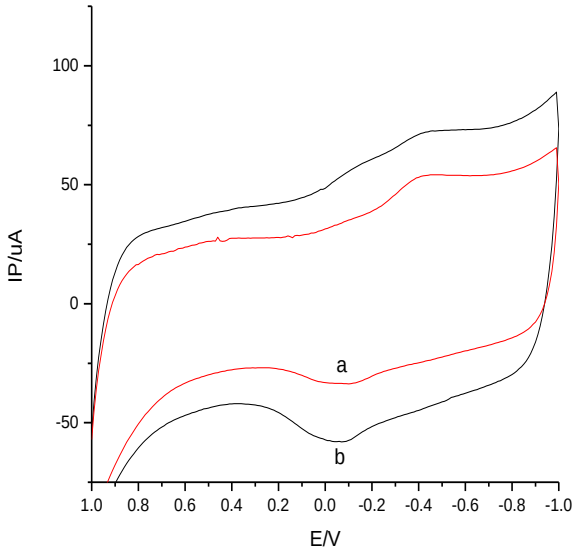

Figure 2. Scavenging effect of lappaconitine on hydroxyl free radical for the experimental sample (a) and the control sample (b).

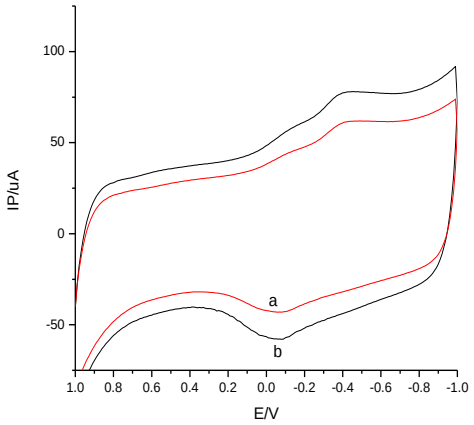

Figure 3. Scavenging effect of HBr-lappaconitine on hydroxyl free radical for the experimental sample (a) and the control sample (b).

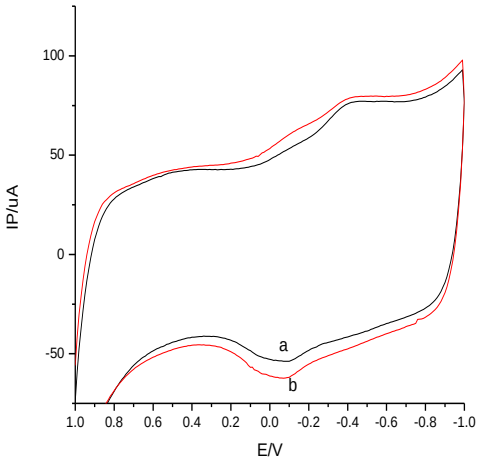

Figure 4. Scavenging effect of HCl-lappaconitine on the hydroxyl free radical for the experimental sample (a) and the control sample (b).

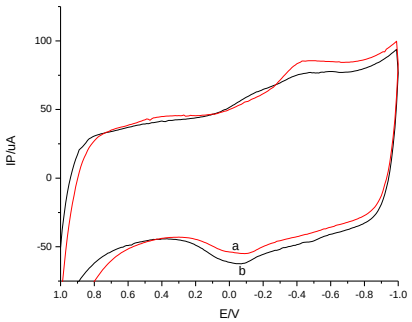

Figure 5. Scavenging effect of lappaconitine on superoxide anion radical for the experimental sample (a)and the control sample (b).

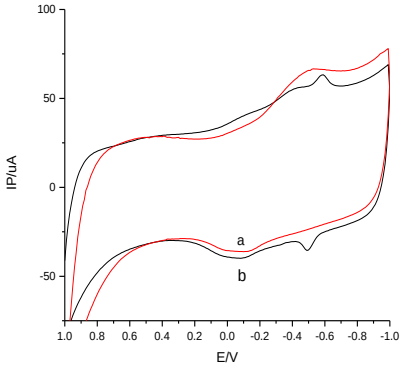

Figure 6. Scavenging effect of HBr-lappaconitine on superoxide anion radical for the (a) experimental sample and (b) the control sample

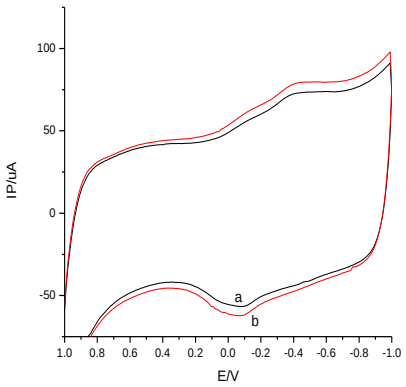

Figure 7. Scavenging effect of HCl-lappaconitine on superoxide anion radical for the experimental sample (a) and the control sample (b).

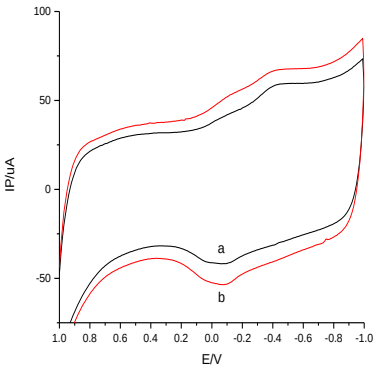

Figure 8. Scavenging effect of lappaconitine on 2,2-diphenyl-l-picrylhydrazyl for the experimental sample (a) and the control sample (b).

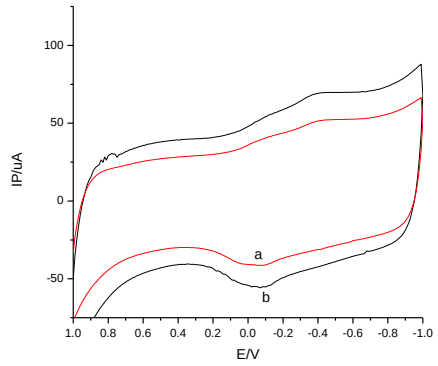

Figure 9. Scavenging effect of HBr-lappaconitine on 2,2-diphenyl-l-picrylhydrazyl for the experimental sample (a) and the control sample (b).

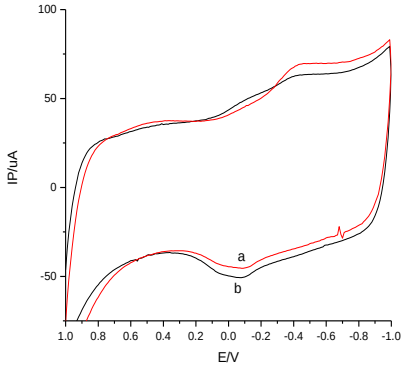

Figure 10. Scavenging effect of HCl-lappaconitine on 2,2-diphenyl-l-picrylhydrazyl for the experimental sample (a) and the control sample (b).

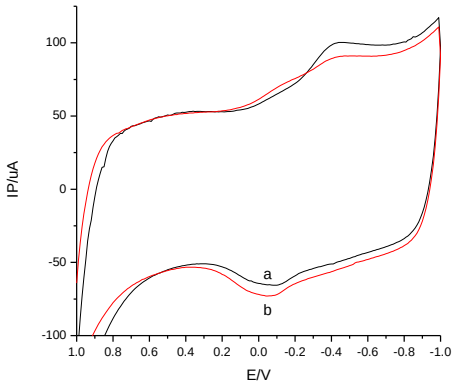

Figure 11. Scavenging effect of lappaconitine on the lipid peroxyl radicals for the experimental sample (a) and the control sample (b).

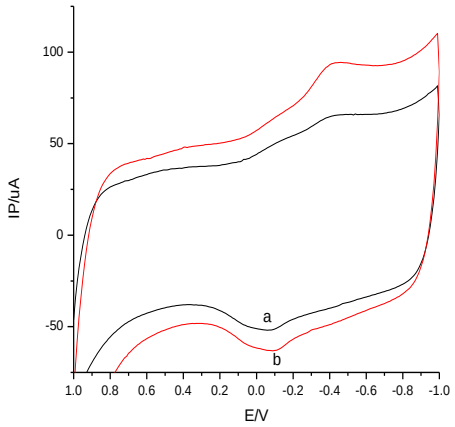

Figure 12. Scavenging effect of HBr-lappaconitine on lipid peroxyl radicals for the experimental sample (a) and the control sample (b).

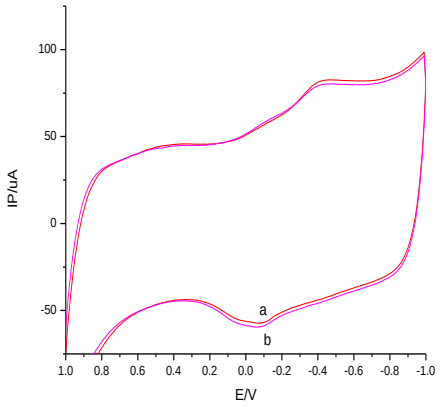

Figure 13. Scavenging effect of HCl-lappaconitine on lipid peroxyl radicals for the experimental sample (a) and the control sample (b).

4. CONCLUSIONS

By using electrochemical analysis with the change in peak current as the contrast parameter, the oxidation resistance of lappaconitine, HBr-lappaconitine and HCl-lappaconitine on MWNT-modified carbon paste electrodes was studied in detail. The conclusions drawn from the data analysis include the following. Lappaconitine, HBr-lappaconitine and HCl-lappaconitine had certain scavenging effects on free radicals. In terms of ranking the effects against free radicals: lappaconitine's ability to scavenge free radicals was 2,2-diphenyl-l-picrylhydrazyl > hydroxyl radical > superoxide anion radical > lipid peroxy radical; HBr-lappaconitine's ability to scavenge free radicals was 2,2-diphenyl-l-picrylhydrazyl > lipid peroxy radical > hydroxyl radical > superoxide anion radical; and HCl-lappaconitine's ability to scavenge free radicals was hydroxyl radical > 2,2-diphenyl-l-picrylhydrazyl > superoxide anion radical > lipid peroxy radical. However, the ability to scavenge 2,2-diphenyl-l-picrylhydrazyl and lipid peroxy radical decreased in the order HBr-lappaconitine >

lappaconitine > HCl-lappaconitine. The ability to scavenge superoxide anion radical and hydroxyl radical decreased in the order lappaconitine > HBr-lappaconitine > HCl-lappaconitine. The results showed that lappaconitine, HBr-lappaconitine and HCl-lappaconitine all exhibit antioxidant activity, although differences were observed. The reported electrode enabled the sensitive, simple, and rapid determination of free-radical scavenging ability. Lappaconitine and its salts can be developed as natural antioxidants.

ACKNOWLEDGMENTS
This work was supported by the Natural Science Foundation of Gansu Province (18JR3RA079), Science and technology program of Gansu Food and Drug Administration (2018GSFDA014), and National Natural Science Foundation of China (21567015).

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