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An Antimicrobial Cerebroside from the Liposoluble Constituent of Cervus Nippon Antler Velvet Layer

Nina Bao[1,*], Yongguang Yin[2] and Panpan Wang[3]

[1]*School of Biological and Food Engineering, Suzhou University, Suzhou 234000, China*
[2]*School of Biological and Agricultural Engineering, Jilin University, Changchun 130022, China*
[3]*School of Foreign Studies, Suzhou University, Suzhou 234000, China*

ABSTRACT

Using petroleum ether to extract an antimicrobial cerebroside from the liposoluble constituent of cervus nippon antler velvet layer was studied in this paper. Single-factor experiment was used to research the effect of liquid-to-solid ratio, standing time and centrifugation time on the extraction of crude cerebroside. The optimal extraction conditions for the cerebroside as follows: liquid-to-solid ratio 15, standing time 45 min, and centrifugation time 6 min. The extracted cerebroside was purified and identified by using silica gel column and TLC, respectively. IR analysis showed that the extraction has hydroxyl and amide groups, which was proved to be cerebroside. Meanwhile, bioactivity showed that the extraction has antibacterial activity, and the purified one is better than the crude one.

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Authors' Contribution
NB designed the study, performed the experimental work, analyzed the data and wrote the article. YY helped in the funds of this study. PW helped in writing.

Key words
Cerebroside, Antler velvet layer, TLC, IR, Bioactivity.

INTRODUCTION

As one kind of traditional Chinese medicine, Cervus nippon antler velvet layer has been widely used in oriental medicine (Zhou and Li, 2009a). The liposoluble constituent of cervus nippon antler velvet layer (sex hormone (Zhou and Li, 2009a; Zhou *et al*., 2009a), phospholipids, p-hydroxybenzaldehyde (Zhou and Li, 2009b; Zhou *et al*., 2009b), uracil, hypoxanthine (Zhou *et al*., 2009b)) which plays a significant role in sexual-reinforcing (Chen *et al*., 1992), anti-aging effect (Zhang *et al*., 1992; Wang *et al*., 1988), and so on is also been widely investigated. But glycolipid (Inagaki *et al*., 2006; Para *et al*., 2009; Mansoor *et al*., 2007; La *et al*., 2012; Ikada *et al*., 2009) was scarce investigated which also belonged to the liposoluble constituent of cervus nippon antler velvet layer.

Glycolipid (cerebroside and ganglioside), particularly cerebroside, has been proved to exhibit various physiological activities, including antitumor/cytotoxic (Natori *et al*., 1994; Jin *et al*., 1994; Li *et al*., 1995; Chen *et al*., 2009), antifungal (Jin *et al*., 1994), immunomodulatory, cyclooxygenase inhibitory, and antifouling activities (Mansoor *et al*., 2007). Thus, it caused me great interest in finding the physiological activities of cerebroside from the liposoluble constituent of cervus nippon antler velvet layer.

* Corresponding author: ninabao1984@163.com
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Generally, using chloroform and methanol to extract cerebroside from the tissues will cause losses in biological activity, so we used supercritical carbon dioxide to extract the liposoluble constituent of cervus nippon antler velvet layer and extracted cerebroside with petroleum ether. Meanwhile, silica gel column was used to purify cerebroside and thin layer chromatography (TLC) was used to identified cerebroside. Characterization of extraction was analyzed by IR (Chen *et al*., 2003; Cateni *et al*., 2003).

The aims of this paper were to (i) use petroleum ether to extract cerebroside from the liposoluble constituent of cervus nippon antler velvet layer, (ii) use silica gel column to isolate and purify cerebroside, (iii) use IR to characterize cerebroside and (iv) use agar diffusion test to evaluate antimicrobial properties.

MATERIALS AND METHODS

Materials

Development College of Agriculture University provided the boiled-dry cervus nippon antler velvet. More than 99.0% purity of the cholesterol standard sample was purchased from the Chemical Company of Sigma (USA).

Supercritical CO_2 extraction

Speed SFE instrument was used to execute the supercritical extraction which was made by Applied Separations Incorporated, Allenton, PA, USA. The

different factors of supercritical carbon dioxide extraction experiment were used to extract the liposoluble constituent of cervus nippon antler velvet layer as follows: the size of particle 250μm, co-solvent ethanol–water (75:25, v/v), temperature of extraction 70°C, and pressure of extraction 30MPa.

Petroleum ether extraction

Weighed five grams of the liposoluble constituent of cervus nippon antler velvet layer, and stirred them to dissolve in the petroleum ether completely. After staying a moment, the cerebroside was obtained by centrifuge at 6000×g. The residue obtained by centrifuge was heated for 6h under the temperature of 40°C *in vacuo* which turned to be a power with amorphism.

Determination of cholesterol

The method of O-phthalaldehyde colorimetric which was described by Xu *et al.* (2006) was used to determine cholesterol contents of the residue.

Separation and purification of cerebroside

The dissolved cerebroside which was obtained by using chloroform and methanol (2:1/v:v) was subjected to column chromatography on silica gel, and then was eluted with chloroform-methanol-H_2O (20:1:0.2), by which 3 fractions (1,2,3) was afforded. Fraction 2 was isolated again to yield cerebrosides by column chromatography on silica gel, and with the method of eluting with chloroform and chloroform-methanol (19:1/v:v), respectively. Chloroform and chloroform-methanol (19:1/v:v) as a solvent to purify the cerebrosides by using column chromatography on silica gel, they were identified by TLC (Cateni *et al*., 2010; Ravneet *et al*., 2010).

Identification of cerebroside

The purified cerebroside was evaluated with TLC. The TLC plates were purchased from the Chemical Company of Qingdao (China). 20 microlitres solution was applied and used chloroform:methanol: water= 10:1:0.2 (v/v) as the solvent system.

Characterization analysis of cerebroside

Using KBr to preparing the sample as pellet, and then scanned it from 4000cm^{-1} to 500cm^{-1} by IRPrestige-21 infrared spectrophotometer, the peaks of which was reported in cm^{-1}.

Antimicrobial properties of cerebroside

Antimicrobial properties of cerebroside were evaluated by *E. coli* which was got from excrement with the method of the minimum inhibitory concentration (MIC) (Cateni *et al*., 2010; Ravneet *et al*., 2010; Liaqat *et al*., 2017). DMSO (20%)(1mL) was used to dissolve dried extraction and purified compound, and obtain a concentration of 8000 and 7000 μg/mL finally. 500μL microbial suspension which grew at 37 °C for an overnight was added to the tube one by one, and subsequently cultured aerobically 24 h under the temperature of 37 °C. MIC will be proved to the lowest concentration of the cerebroside if there were color change (red to yellow) or visible growth because with the microbial growth, it will form acidic metabolites.

RESULTS AND DISCUSSION

Effect of liquid-to-solid ratio on the weight of residue

The liquid-to-solid ratio, which directly influences the purity of crude cerebrosideis, has great effect on the residue of cerebroside extraction. As shown in Figure 1, the amount of residue of cerebroside extraction decreased from 0.402±0.007g to 0.076±0.016g when the liquid-to-solid ratio increased from 5 to 19. The cholesterol which was retained in crude cerebroside varied from 0.017±0g to 0.008±0g. This is so obvious that the more petroleum ether the less residue, which is because more petroleum ether caused more dissolution of fatty acid which resulted in less residue, therefore made crude cerebroside more pure. When the liquid-to-solid ratio changed from 15 to 19, the weight of cerebroside decreased steadily, this indicated that the free fat acid almost dissolves in petroleum ether. The present study chose the liquid-to-solid ratio at 15 to extract the cerebroside.

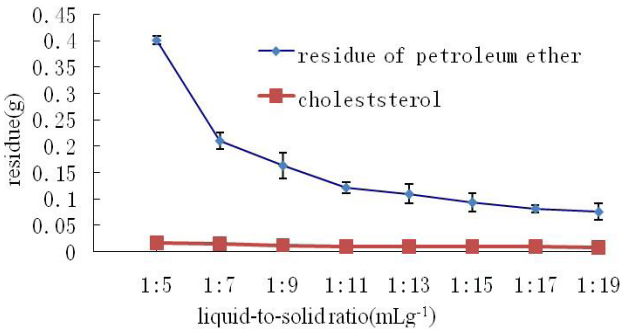

Fig. 1. Effect of liquid-to-solid ratio on the weight of residue.

Effect of standing time on the weight of residue

Standing time also plays a significant role for the residue of cerebroside extraction. Figure 2 revealed that as the standing time changed from 15 min to 75 min, cerebroside decreased from 0.108±0.010g to 0.086±0.001g. The cholesterol which was retained in crude cerebroside varied from 0.010±0.002g to 0.011±0g, with

little variation with the standing time. When the standing time reached 45 min, the weight of cerebroside changed little and the curve became smooth. With the increase of standing time, it can be seen that more fatty acid dissolves in petroleum ether and reach saturation. So the weight of cerebroside will decrease and then turn to be constant. The weight of cerebroside decreases until it comes to 45 min, therefore this study chosen standing time at 45 min as a precondition to carry on the experiment.

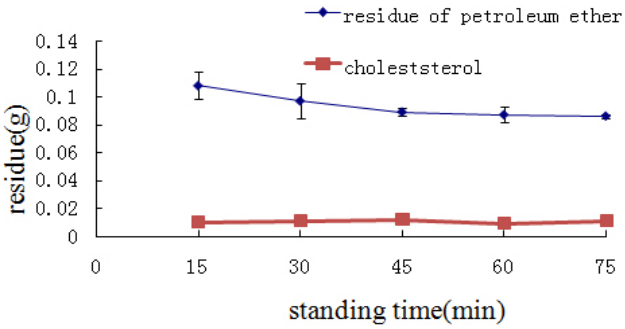

Fig. 2. Effect of standing time on the cerebroside extraction.

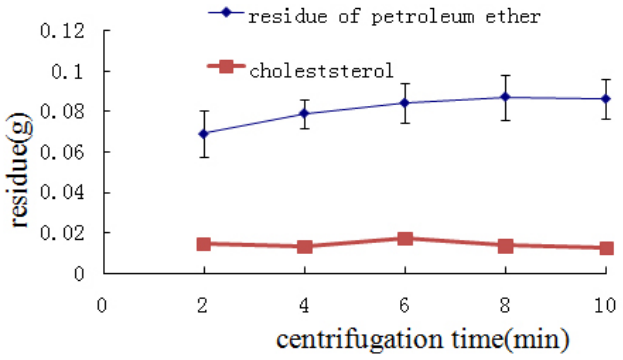

Fig. 3. Effect of centrifugation time on the cerebroside extraction.

Effect of centrifugation time on the weight of residue

Figure 3 revealed that centrifugation time affects the weight of residue. As the time increases from 2 min to 10 min, the weight of cerebroside increases from 0.069±0.011g to 0.086±0.010g. The cholesterol which was retained in crude cerebroside varied from 0.015±0.001g to 0.013±0.001g. It's true that the more centrifugation time the more weight of cerebroside, when it comes to 6min, the change trend of the cerebroside weight becomes smooth. Therefore, the centrifugation time at 6min was favorable for this study.

Identification of cerebroside

Figure 4 indicated that TLC was used to identify the residue, which was extracted with petroleum ether. Ethanol-H_2SO_4-spray method was adopted to visual the cerebroside and GF 254 (20×20) was used to carry out TLC. 5% aq. H_2SO_4 was firstly used to spray the sample, which then was heated at 110°C for 5mins until the brownish yellow trace appeared on the white background. Lane 1 was crude cerebroside, Lane 2 was eluted with chloroform, while Lane 3 was eluted with chloroform-methanol 19:1, and Lane 4 stood for the cholesterol standards. One spot means pure on silica gel plate of TLC. The results showed that when chloroform was used to elute, only cholesterol can be obtained, while chloroform-methanol 19:1 was adopted to elute, only cerebroside can be acquired which signifies the purify of cerebroside.

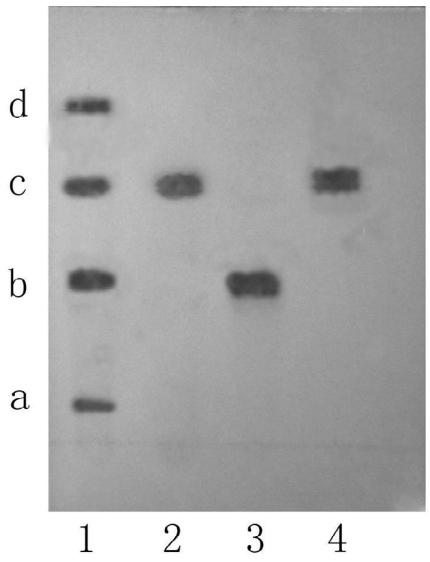

Fig. 4. TLC of cerebroside in the extracts. 1, the crude cerebrosdie; 2 and 3, constituent eluted with chloroform and chloroform-methanol (19:1/v:v), respectively; 4, standard of cholesterol.

IR analysis of cerebroside

IR absorbance peaks at 3311 cm^{-1}, 1642 cm^{-1} and 1550 cm^{-1} (Fig. 5) were in consistent with absorbance peaks of hydroxyl and amide groups.

As the infrared characteristic absorption peaks of cerebroside are hydroxyl and amide groups, it is possible to infer cerebroside. The IR absorptions which reported by Chen *et al*. (2003) showed that hydroxyl and amide groups were 3323 cm^{-1}, 1661cm-1 and 1557 cm^{-1}, respectively; and as reported by Cateni *et al*. (2003), the IR absorbance peaks of hydroxyl and amide groups were 3413cm^{-1}, 1646cm^{-1} and 1540 cm^{-1}, respectively. In conclusion, residue which was extracted from the liposoluble constituent of cervus nippon antler velvet layer was confirmed to be cerebroside.

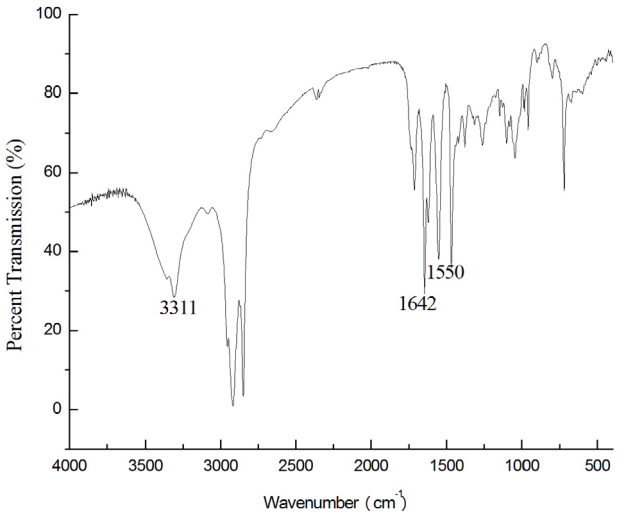

Fig. 5. IR spectrum of cerebroside.

Antimicrobial properties

The microbroth dilutioin method (against *Staphylococcus aureus* and *Escherichia coli*) was used to evaluate the biological activity of the cerebroside which was extracted from the liposoluble constituent of cervus nippon antler velvet layer. Results showed that cerebroside inhibited growth of *E. coli*. Meanwhile the minimum inhibitory concentrations of the purified one and the crude one were 27μg/mL and 125μg/mL, respectively.

The known cerebrosides isolated from *Euphorbia peplis* L. and *Pinellia ternate* showed good antimicrobial activities on Gram positive bacterium (*S. aureus*), both of them were not inhibitory to the Gram negative bacterium (*E. coli*). However, the biological activities of cerebroside from cervus nippon antler velvet showed no activity against *S. aureus*, but it worked on *E. coli*. In conclusion, the purified cerebroside and crude cerebroside isolated from the liposoluble constituent of cervus nippon antler velvet layer showed inhibitory activities on *E. coli*, but had no biological activities on *S. aureus*. Meanwhile, the purified cerebroside showed better than the crude cerebroside.

CONCLUSIONS

This study used petroleum ether to investigate the extraction of cerebroside from the liposoluble constituent of cervus nippon antler velvet layer. The liquid-to-solid ratio 15, standing time 45 min, and centrifugation time 6min were the conditions for cerebroside extraction. The cerebroside was purified by column chromatography of silica gel eluting with chloroform and chloroform-methanol (19:1/v:v), which was identified by TLC. IR analysis showed the residue contained hydroxyl and amide groups, which are the characteristic absorption peaks of cerebrosides and can be used to testify the residue, which is cerebroside. The purified cerebroside showed better antimicrobial properties than the crude one, and the MIC of them were 27μg/mL and 125μg/mL, respectively. The cerebroside had inhibitory activities on *E. coli*, but had no biological activities on *S. aureus*.

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Statement of conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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