Diuretic Ingredients of *Poria cocos*

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**ABSTRACT**

To determine the diuretic ingredients of *Poria* and reveal the underlying action mechanisms, multi-mode separation methods were employed to split the different fractions of *Poria* to yield Petroleum Ether Fraction (PEF), Ethyl Acetate Fraction (EAF), Water Eluted Fraction (WEF), Alcohol Eluted Fraction (AEF), Crude Polysaccharide Fraction (CPF) and Refined Polysaccharide Fraction (RPF). The rats were orally administered of the water decoction and different splitted fractions of *Poria*, purified water was used as control. Urine samples were collected to determine the concentration of Na⁺, K⁺ and Cl⁻ and the blood samples were collected to determine the urine creatinine (Ucr), serum creatinine (Scr) and Na⁺-K⁺ ATPase. Furthermore, kidney tissues were also collected to test the Na⁺-K⁺ ATPase and Carbonic Anhydrase (CA). The results showed that the volume of urine and the concentration of Na⁺, K⁺ and Cl⁻ in the 6 h urine samples were dramatically increased in the rats administered i.e., with water decoction and EAF as compared to the control group. Furthermore, the water decoction of *Poria* showed inhibitory effects on Na⁺-K⁺ ATPase in kidney while EAF showed inhibitory effects on the Na⁺-K⁺ ATPase action in both blood and kidney. Therefore, the EAF should be the diuretic fraction of *Poria*. The underlying diuretic action mechanisms of the EAF might be due to: (1) Increasing the release of Na⁺ and Cl⁻ in urine and (2) Inhibiting the Na⁺-K⁺ ATPase activity in both blood and kidney.

**Key words:** Diuretic action, fraction, *Poria cocos* (Schw.) wolf, *Poria*

**INTRODUCTION**

*Poria* (or Fu-Ling in Chinese), the dried sclerotium of *Poria cocos* (Schw.) Wolf (Chinese Pharmacopoeia Commission, 2010), is a fungus of the Polyporaceae family which has been used clinically for about 2000 years in China. *Poria* is mainly found in Yunnan (also called Yunling), Anhui (also called Anling), Guizhou, Guangxi, Hubei, Fujian (also called Minling) of China and it exhibits sweet and pale in flavor, mild in nature in accordance with theory of Traditional Chinese Medicine (TCM). It was recorded that *Poria* was specialized in diuretic action, invigorating the spleen and tranquilizing the mind in the Chinese Pharmacopoeia Commission (2010).

It was reported that triterpenoids, polysaccharides and fatty acids are the main ingredients in *Poria*. Tetracyclic triterpenoids mainly include pachymic acid, tumulosic acid, 3β-hydroxy lanosta, pachymic acid methyl ester, tumulosic acid methyl ester, polyenonic acid C methyl ester and so on. Polysaccharide mainly composed of pachymarin, pachymaran and gluvin H11 (Hu et al., 2006; Yang et al., 2014; Zheng and Yang, 2008a, b). Modern pharmacological studies indicated that *Poria* possessed the activity of diuresis, sedative, antioxidant, anti-hyperglycaemia, anti-bacterial, anti-tumor, hepatoprotective and immunomodulating actions (Niu et al., 2012; Zhong and Liu, 2001; Huang and Zhang, 2011; Zhang et al., 2004; Lee et al., 2012). To elucidate the effective components of various pharmacological actions of *Poria*, previously, the fraction splitting and their overlapping degrees was studied (Lin et al., 2013). Continuously, this study dealt with the diuretic components of *Poria* and their action mechanisms.
MATERIALS AND METHODS

Drugs and reagents: *Poria* was collected from Tengchong, Yunnan, China, November 2012 (Batch No. 20121101), where is geo-authentic producing area and were identified by Professor Wangbing (Liaoning University of Traditional Chinese Medicine) as a fungus of the Polyopostaceae family. Hydrochlorothiazide tablets (Batch No. 1212029, Tianjin Lisheng Pharmaceutical Co. Ltd). Rat CA Elisa Kit (Batch No. 201310, R and D), ultramicro ATPase assay kit (Batch No. 20130123, Nanjing Jiancheng Bioengineering Institute), ATPase assay kit (Batch No. 20131024, Nanjing Jiancheng Bioengineering Institute), Quantichrom™ Hemoglobin Assay Kit (Batch No. 2013022, Changchun Huili Biotech Co. Ltd), Coomassie blue assay kit (Batch No. 20130117, Nanjing Jiancheng Bioengineering Institute). Normal saline was obtained from Dubang Pharmaceutical Ltd (Batch No. 1303090307, Jilin, China). Other reagents were of analytical grade.

Animals: Male Sprague Dawley (SD) rats (240–260 g) were bought from Liaoning Changsheng Biotechnology Co., Ltd [SCXXK (Liao) 2010-0001]. All rats were maintained with free access to food and water in plastic cages at 22±2°C, relative humidity 50–60% and kept on a 12 h light/dark cycle. Animals were housed for one week prior to the experiments. The experimental protocols were approved by the Guide for the Care and Use of Laboratory Animals Published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All studies were carried out in accordance with the ethics regulations of Liaoning University of TCM (131/2010).

Preparation of split fractions: Three hundred gram of *Poria* was pulverized and immersed in water for 1 h, boiled twice and the filtrates were combined and concentrated in vacuum to 1.2 g crude drug mL⁻¹ and then stored in 4 for the water decoction of *Poria*. Further, according to the polarity of components of *Poria*, the water decoction of *Poria* was split as reported in Lin et al. (2013). Briefly, the water decoction of *Poria* was concentrated and the 95% ethanol was added to regulate the alcohol concentration to 75%, standstill under 0–5 overnight and then the supernatant was poured out to obtain the precipitate and the supernatant. The precipitate was washed three times by 95% ethanol and acetone respectively to furnish the Crude Polysaccharides Fraction (CPF) after lyophilization. Then, the supernatant was evaporated on water bath at 50°C to eliminate the alcohol and the resulting water layer was extracted eight times with 60–90 petroleum ether until there is no color in layer of petroleum ether to give Petroleum Ether Fraction (PEF) and then, the water layer was partitioned with ethyl acetate to yield Ethyl Acetate Fraction (EAF). The resulting water layer was subjected to a column of macroporous adsorption resin D101, washing with distilled water, 80% ethanol and 95% ethanol successively. The water eluate was concentrated at 50 and lyophilized to give Water Eluted Fraction (WEF) and the 80 and 95% ethanol elutes were collected and evaporated in vacuo to give the Alcohol Eluted Fraction (AEF). The CPF was further purified by seavage and enzyme method to furnish the Refined Polysaccharide Fraction (RPF). In addition, based on the HPLC fingerprints of PEF, EAF, AEF and WEF, their overlapping degrees were evaluated to be less than 10%. Over 20 compounds were isolated and identified from the above fractions and thereof, it can be concluded that PEF mainly contains aromatic esters, EAF mainly contains triterpenoids, WEF mainly contains small molecular saccharides, AEF mainly contains amino acid and indole compounds, CPF mainly contains crude polysaccharides, RPF mainly contains refined polysaccharides whose protein was removed from CPF.

Animals prescreening: The animal prescreening was performed according to Aston method (Wang et al. 2006). The rats were placed in the metabolism cage for 3 days, after deprived of food but not water for 18 h. After the rats were administered i.g., of 25 mL kg⁻¹ saline, urine was collected for the first 2 h, the urine volume of the rats over 40% of the oral volume can be selected for the diuretic experiment.

Diuretic activity of *Poria* water decoction: Before the experiment, the rats were deprived of food but not water for 18 h and were randomly divided into 5 groups with 10 rats for each group. The rats were administered i.g., of 40 mg kg⁻¹ saline and 20 min later, each group was administered i.g., as follows. Control group: The 1 mL 100 g of the purified water, positive control group: The 0.01 g kg⁻¹ hydrochlorothiazide, 1.5 g kg⁻¹ WD group: The 1.5 g kg⁻¹ water decoction, 6 g kg⁻¹ WD group: The 6 g kg⁻¹ water decoction, 12 g kg⁻¹ WD group: The 12 g kg⁻¹ water decoction. After *Poria* was administered at 1 and 7 day, the rats were immediately put into the metabolism cage and the urines were collected every 2 h in total 6 h.

Diuretic action of the split fractions of *Poria*: The experiment protocol is similar to the water decoction of *Poria* and the rats were randomly divided into 9 groups with 10 rats for each group. The rats were firstly administered i.g., with 40 mL kg⁻¹ saline and 20 min later, each group was orally administered i.g., as follows. Control group. The purified water 1 mL 100 g, positive control group: The 0.01 g kg⁻¹ hydrochlorothiazide, 6 g kg⁻¹ WD group: The 6 g kg⁻¹ water decoction and the 6 split fractions: Corresponding extracts according to 6 g kg⁻¹ crude herbs. After *Poria* was administered at 1 and 7 day, the rats were immediately put into the metabolism cage and the urines were collected every 2 h in total 6 h.

Determination of Na⁺, K⁺ and Cl⁻ in urines and Ucr: After urine collection, the rats were anaesthetized and then, blood samples were collected from retro ocular venous plexus of rats. Serum and erythrocytes were separated for the analysis of Sore and Na⁺-K⁺ ATPase. Finally, the rats were sacrificed and their kidneys were isolated. The kidney was homogenized for the determination of Na⁺-K⁺ ATPase and CA.
The concentrations of Na⁺, K⁺ and Cl⁻ in urines and Ucr and Scr of serum were determined by Liaoning Provincial Hospital of Traditional Chinese Medicine.

The level of Na⁺-K⁺ ATPase were determined according to the indication ATPase assay kit.

**Statistical analysis:** All data were expressed as the Mean±Standard deviation with the use of SPSS19.0 software. All indexes were analyzed by analysis of variance. The p-value less than 0.05 was considered to be statistical significance.

**RESULTS**

**Diuretic effects of *Poria* water decoction in rats:** Results (Fig. 1) indicated that the urine volume had no significant difference after administered once with different doses of water decoction of *Poria*. However, the one week administration with *Poria* water decoction showed that the middle dose of *Poria* showed dramatically diuretic action while the low and high doses of the water decoction of *Poria* could not show obvious diuretic action.

**Diuretic fractions of *Poria* in rats:** The water decoction of *Poria* was split into PEF, EAF, WEF, AEP and CPF as well as RPF. Diuretic test (Fig. 2) indicated that urine volume of the EAF group increased obviously during the first 4 h after administered once with the splitted fractions. Further, 7 days administration with *Poria* and its splitted fractions showed that the urine excretion increased obviously in both water decoction and the EAF groups of *Poria*, especially the first 4 h after drug administered. Although the urine output in polysaccharides showed a little increment, there was no obvious statistical difference as compared with control group.

**Effects of *Poria* on Na⁺, K⁺ and Cl⁻ of urine:** The results (Table 1) revealed that the releasing of Na⁺ was dramatically increased in EAF, CPF and RPF groups and the releasing of K⁺, Cl⁻ were also significantly increased in each group.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Na⁺ (mmol L⁻¹)</th>
<th>Na⁺ (mg)</th>
<th>K⁺ (mmol L⁻¹)</th>
<th>K⁺ (mg)</th>
<th>Cl⁻ (mmol L⁻¹)</th>
<th>Cl⁻ (mg)</th>
<th>Na⁺/K⁺</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>51.65±13.49</td>
<td>9.26±3.076</td>
<td>44.38±13.01</td>
<td>13.09±1.182</td>
<td>56.07±14.78</td>
<td>15.58±5.190</td>
<td>0.708±0.1422</td>
</tr>
<tr>
<td>6 g kg⁻¹ WD group</td>
<td>60.64±11.67</td>
<td>13.12±3.879</td>
<td>53.53±12.28</td>
<td>18.94±3.127***</td>
<td>73.60±13.59**</td>
<td>24.25±5.856***</td>
<td>0.69±0.0237</td>
</tr>
<tr>
<td>WEF</td>
<td>60.93±19.06</td>
<td>8.38±3.139</td>
<td>62.09±9.420**</td>
<td>14.58±3.074</td>
<td>85.48±13.82***</td>
<td>18.30±4.555</td>
<td>0.58±0.02193</td>
</tr>
<tr>
<td>AEF</td>
<td>60.77±12.27</td>
<td>9.19±2.744</td>
<td>61.85±11.18***</td>
<td>15.94±6.618</td>
<td>78.24±12.34***</td>
<td>18.39±5.14</td>
<td>0.59±0.1718</td>
</tr>
<tr>
<td>PEF</td>
<td>64.76±10.80</td>
<td>12.86±4.171***</td>
<td>62.40±8.155***</td>
<td>20.70±5.619***</td>
<td>80.19±10.59***</td>
<td>24.22±6.460***</td>
<td>0.61±0.1095</td>
</tr>
<tr>
<td>EAF</td>
<td>79.53±10.36***</td>
<td>17.66±5.141***</td>
<td>68.33±8.166***</td>
<td>25.22±5.005***</td>
<td>95.18±13.88***</td>
<td>32.52±9.416***</td>
<td>0.69±0.1097</td>
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<tr>
<td>CPF</td>
<td>74.08±10.48***</td>
<td>15.29±3.655***</td>
<td>70.56±12.64***</td>
<td>24.81±7.376***</td>
<td>85.07±16.34***</td>
<td>27.08±7.432***</td>
<td>0.62±0.0808</td>
</tr>
<tr>
<td>RPF</td>
<td>79.50±11.54***</td>
<td>16.09±4.496***</td>
<td>73.48±17.70***</td>
<td>24.48±5.074***</td>
<td>94.21±20.99***</td>
<td>28.84±6.714***</td>
<td>0.66±0.1699</td>
</tr>
<tr>
<td>Positive control</td>
<td>78.36±14.44***</td>
<td>19.61±4.113***</td>
<td>50.68±12.09</td>
<td>21.20±5.363***</td>
<td>95.62±23.59***</td>
<td>36.80±9.097***</td>
<td>0.94±0.1388***</td>
</tr>
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Values are the Mean±SD in LSD analysis, *p<0.05, **p<0.01 compared with control group.

**Fig. 1(a-b):** Time courses of diuretic activities for (a) 1 day administration and (b) 7 day administration. The volume of excreted urine was measured at 2, 4 and 6 h after administration of *Poria*. Cumulative values are reported as Mean±SE for 10 rats in each group. *p<0.05 and **p<0.01 compared to controls using LSD analyse.
Fig. 2(a-b): Time courses of diuretic activities for (a) 1 day administration and (b) 7 day administration. The volume of excreted urine was measured at 2, 4 and 6 h after administration. Cumulative values are reported as Mean±SE for 10 rats in each group. *p<0.05 and **p<0.01 compared to controls using LSD analyse.

Fig. 3(a-b): Effects of different fractions of *Poria* on (a) Na⁺-K⁺-ATPase, Na⁺-K⁺-ATPase in erythrocytes and (b) Na⁺-K⁺-ATPase in kidney. Cumulative values are reported as Mean±SE for 10 rats in each group. *p<0.05 and **p<0.01 compared to controls using LSD analyse.

**Effect on Na⁺-K⁺-ATPase level of blood and kidney:** In Fig. 3, the results disclosed that the WEF, AEF, PEF and CPF possessed the ability to increase the Na⁺-K⁺ ATPase level of erythrocytes. However, EAF decreased the level of erythrocytes Na⁺-K⁺ ATPase which was similar to the positive control group. EAF also decreased the level of kidney Na⁺-K⁺ ATPase, indicating that inhibitory effect on the activity of Na⁺-K⁺ ATPase may be one of the reason for the diuretic action of *Poria*. 

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Effects of different fractions of *Poria* on Scr and Ucr: From the results shown in Fig. 4, there were no significant changes in Scr after administration with each splitted fraction. However, endogenous creatinine clearance was dramatically increased by CPF group (p<0.01) and this effect was also observed in EAF, AEF and WEF groups but a little weaker (p=0.05).

Effects of different fractions of *Poria* on the level of CA in kidney: The result as in Fig. 5, indicated that only AEF showed significant difference as compared with control, indicating that AEF may inhibit the diuretic action.

**DISCUSSION**

Since, the plant living organisms contains thousands of components, just a few individual compounds always could not represent the whole pharmacologic effects. In this study, we attempted to use splitted fraction obtained from the crude extract overcome this disadvantages. Multi-mode splitting
methods consisted of solvent partition method and chromatography were used to give 6 fractions which overlapping degrees were evaluated to be less than 10%, to overcome the problem that the same component is found in several crude extracts. As for the dosage, in order to reflect the real effective material of *Poria*, in this study, the minimum dose of *Poria* water decoction for diuretic action was firstly explored and then the doses of the splitted fractions were determined with corresponding recovery rates.

Our result showed that the water decoction possessed no diuretic activity after first administration which is consistent with Tian *et al.* (2014). However, the urine volume of the middle dose of *Poria* water decoction was dramatically increased. Therefore, the middle dose was selected for further study. Furthermore, EAF showed stronger activity in urine excretion in both 1 and 7 days administration, enlightening that EAF is the active fraction for the diuretic action of *Poria*.

Hydrochlorothiazide is a widely used diuretics, its mechanism is to inhibit the reabsorption of Na\(^+\) and Cl\(^-\). However, certain side effects were observed, such as low level of blood potassium. In the current study, water decoction and EAF possessed the diuretic action but the action mechanisms were different from hydrochlorothiazide that it can modulate the excretion of K\(^+\) to perform its diuretic action. Pharmacological studies have confirmed that tetracyclic triterpenoids of *Poria* can bind to renal cytoplasmic aldosterone receptors in an *in vitro* experiment and have an anti-aldosterone activity and enhance the urinary Na\(^+\)/K\(^+\) ratio with dose-dependence in rats (Deng and Xu, 1992). Thus, the diuretic action of EAF may be related to this mechanism.

In addition, it is the first time that we found the Na\(^+\)-K\(^+\) ATPase of kidney may be one of the factor causing the diuretic effect of *Poria*. The urine formation process included three procedures, i.e., the glomerular filtration, the reabsorption in the renal tubule and collecting tubule and the excretion of tubule and collect tubule. The Na\(^+\)-K\(^+\) ATPase of erythrocyte could affect the water level in the blood and change the water volume which was filtered by the glomerular capillary to form crude urine. When the crude urine flowed through the renal tubule and collecting duct, partial water and ions (Na\(^+\), K\(^+\), etc.) were reabsorbed. The remaining part entered bladder and the urine formed. Thus, if the activity of Na\(^+\)-K\(^+\) ATPase of erythrocyte was decreased, crude urine could be increased. The inhibitory effect on the Na\(^+\)-K\(^+\) ATPase of kidney could decrease the reabsorption of crude urine and urine excretion will increase. EAF showed inhibitory effect on the Na\(^+\)-K\(^+\) ATPase activity in both erythrocyte and kidney, indicating this is another way of EAF to cause diuretic action. The decreasing of Na\(^+\)-K\(^+\) ATPase level in kidney may be caused by the tetracyclic triterpenoid components of EAF which can bind to renal cytoplasmic aldosterone receptors. As indicated by Loew *et al.* (1991), the polar components increase renal circulation and thus the rate of glomerular filtration that promotes increased urine formation.

CA is a group of enzymes containing zinc which participate in the reabsorption of HCO\(_3^-\) in the kidney. Our results indicated that AEF could activate CA level of kidney, indicating AEF possessed definite anti-diuretic action. The water decoction contained diuretic components of EAF and anti-diuretic ones of AEF, so the diuretic action of water decoction is weaker than EAF.

Water decoction and EAF showed no activities in Scr and Ucr which indicated that *Poria* and its fractions, could not affect the physiological function of kidney, indicating that *Poria* possessed the diuretic action with fewer side effects. These data suggested that *Poria* is a safe and effective diuretic drug. Thus, EAF should be the diuretic fraction of *Poria* and its other actions related with the traditional use of *Poria* are still in exploring.

**ACKNOWLEDGMENTS**

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