Analysis of Maceaene and Macamide Contents of Petroleum Ether Extract of Black, Yellow, and Purple *Lepidium Meyenii* (Maca) and Their Antioxidant Effect on Diabetes Mellitus Rat Model

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ABSTRACT

Maceaene and macamide contents as well as antioxidant effect of petroleum ether extract of black maca (BM), yellow maca (YM), and purple maca (PM) on diabetes mellitus (DM) rats were investigated. The results showed that seven, six, and five analogues of macamides were identified from the petroleum ether extracts of BM, YM, and PM, respectively. BM extract exhibited the highest contents of total macamides. Comparatively, the PM extract has the lowest macamide quantity. The maceaene contents in all the extracts showed no significant difference (p>0.05). Macamide contents in maca with the same color were not statistically different. Pharmacological results showed that 60-day oral administration of the petroleum ether extract of maca (100 mg/kg.d) can significantly decrease lipid oxidation as indicated by the decreased thiobarbituric acid reactive substances (TBARS) and carbonylated proteins (CP) concentrations on DM rat model (P<0.05). Among them, oral administration of PM extract showed the lowest TBARS and CP concentrations. All maca extracts can enhance antioxidant enzyme (SOD, superoxide dismutase; CAT, catalase) activity of liver and red blood cells (RBC) of DM rat. However, only oral administration of PM extract can increase SOD and CAT activity of both RBC and liver. The glutathion (GSH) contents in plasma were significantly increased in DM rats treated with PM extract (p<0.05). But, oral administration of BM and YM extracts did not enhance GSH levels. Take together, the data suggested that PM extract exhibited the most potent antioxidant activity on DM rat model. And, maceaene and macamide in maca extract was not correlated with its antioxidant ability.

**Key words:** maca extract, antioxidant activity, maceaene, macamide, diabetes mellitus rat

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INTRODUCTION

In diabetes mellitus (DM), chronic hyperglycaemia produces multiple biochemical sequelae, and diabetes-induced oxidative stress could play a role in the symptoms and progression of the disease [1]. Oxidative stress may result in overproduction of oxygen free-radical precursors and/or decreased efficiency of the antioxidant system [2]. The oxygen free-radical generation is associated with auto-oxidation of glucose, impaired glutathione metabolism, alterations in the antioxidant enzymes and formation of lipid peroxides [3–5]. There are various endogenous defense mechanisms against free radicals, such as the enzymes GSH, SOD, GPx, and CAT, whose activities eliminate superoxide, hydrogen peroxide and hydroxyl radicals [6]. Oxidative stress is increased in experimental models of streptozotocin (STZ)-induced diabetes mellitus [7].

Maca (Lepidium meyenii Walp. synonym, L. peruvianum Chacon, Brassicaceae), a root crop similar to radish (Raphanus sativus L.), has been consumed in Peru for thousands of years as food and medicine [8]. The plant is originally used to increase human and livestock stamina and to ameliorate fertility problems associated with living at the high elevations where the plant grows [9]. The reputation of maca as a fertility enhancer has increased the popularity of the plant in the USA and other Western countries [10]. Recent studies [11–15] have confirmed the effectiveness of maca as a libido and fertility enhancer. Maca, however, has many other traditional therapeutic uses, e.g. as a laxative and for the treatment of rheumatism, respiratory problems, premenstrual discomfort, and menopausal symptoms [10]. Consumption of maca is also reputed to regulate hormone secretion, stimulate metabolism, improve memory and combat depression, anaemia, leukaemia, AIDS, cancer and alcoholism [16]. Even with the reputation of maca as a panacea, scientific research has primarily been focused on its properties of sexual stimulus. Maca contains several kinds of secondary metabolites [17,18], of which macaene and macamides were mainly and uniquely two types of secondary metabolites in this plant and were often thought to be related its pharmacological properties [11]. It has been observed that maca batches from different producers significantly vary in the amount of macaene and macamides [19–21]. More recently, it was revealed that maca with different skin colors was pharmacologically different [22], which may be led due to the diversity of secondary metabolites.

Yunnan is a part of Yun-Gui plateau with an average altitude of more than 2000 m. Maca, as a herbal medicine, was widely cultivated in the region. The skin colors of maca in the region were mainly black, yellow, and purple. The purpose of this study is to analyze the macaene and macamides of petroleum ether extract of maca in Yunnan Province and evaluate their antioxidant effect on DM rat model.

MATERIALS AND METHODS

Materials

Acetonitrile, reagent methanol, petroleum ether, and trifluoroacetic acid were HPLC grade and purchased from Fisher (USA). Authentic compounds were purchased from Institute of Processing Engineering, Chinese Academy of Sciences (Beijing, China). Identity and purity of each compound were confirmed by chromatographic (TLC and HPLC) and spectroscopic methods (IR, 1D- and 2D-NMR, high resolution electrospray ionization mass spectroscopy (HR-ESI-MS)). Streptozotocin, citrate, and 2-(4-iodophenyl)-3-(4-nitropheno1)-5-phenyltetrazolium chloride were purchased from sigma Co.. Nine samples of maca (three samples each color) were collected in Traditional Chinese Medicine Market of Juhua Village in Kunming City, Yunnan Province, on 8, Sep., 2014. Three samples with black skin were designated as BM1, BM2 and BM3. In a similar way, YM1, YM2, YM3 and PM1, PM2, PM3 referred to maca with yellow and purple skins, respectively.

Sample Preparation

A 1.0 kg portion of the dried plant material was extracted three times with 4.5 L of methanol by sonication for 5 h. The extract was evaporated to get a residue (78-92 g) under reduced pressure, which was suspended in water (80 ml) and then partitioned with petroleum ether. Petroleum ether extract was evaporated to get a dried residue (28–47g). Petroleum ether extract (1g) were dissolved in 50 ml methanol/H₂O (8:2.v/v) for HPLC-MS analysis. The remaining extract was used for animal experiment.
Analytical method
HPLC experiments were performed on a Waters Alliance 2690 HPLC system equipped with a 996 photodiode array detector (Waters, Milford, MA, U.S.A.). A Syngeri MAX-RP 80A column (15034.6 mm, 4mm particle size) from Phenomenex (Torrance, CA, U.S.A.) was used for all separations. The mobile phase consisted of H$_2$O (A) and acetonitrile (B), both containing 0.025% trifluoroacetic acid (TFA). Separations were performed by linear gradient elution from 45A/55B to 5A/95B during 35 min. The flow rate was adjusted to 1.0 mL/min, with detection wavelengths of 210 nm and 280 nm, respectively. The column oven was set to 40°C. 10 mL of sample was injected for singlet analysis by automatic sampler. Peaks were assigned by spiking the samples with standard compounds, and comparison of the UV-spectra and retention time. All data was recorded and processed by Millennium 32 software from Waters.

Experimental design
One hundred (diabetic) and ten (normal) rats divided into eleven groups of 10 animals each were used to investigate the antioxidant effect of maca extracts: normal control group (N-CK), diabetic control group (D-CK), BM1 group (diabetic + 100 mg/kg.d BM1), BM2 group (diabetic + 100 mg/kg.d BM2), BM3 group (diabetic + 100 mg/kg.d BM3), YM1 group (diabetic + 100 mg/kg.d YM1), YM2 group (diabetic + 100 mg/kg.d YM2), YM3 group (diabetic + 100 mg/kg.d YM3), PM1 group (diabetic + 100 mg/kg.d PM1), PM2 group (diabetic + 100 mg/kg.d PM2), and PM3 group (diabetic + 100 mg/kg.d PM3). The extracts were suspended in CMC (0.05 g/ml) and orally administered via an intragastric tube (2 mL/rat) on a daily basis for 60 days. N-CK and D-CK rats were received CMC alone. After the last treatment, all the rats were fasted overnight and sacrificed by cervical decapitation. For each group, levels of plasmatic glucose were determined at the beginning of the experiment, 96 hours after STZ injection and at the end of the experiment. The study was approved by the Ethics Committee of South China Agricultural University (Guangzhou, China) and conformed to the standards set by the Guangdong Experimental Animal Management Board.

Tissue collection and processing
Animals were sacrificed by decapitation after blood collection. Both testes were immediately removed and placed in a Petri dish. After a brief wash in cold saline, fats connective tissues and blood vessels were removed. The liver was homogenized by using a Potter-Elvejhem homogenizer with physiological serum, and centrifuged at 5,000 rpm, then the supernatants were collected for the measurement of biochemical parameters.

Thiobarbituric acid reactive substances assay
Frozen testes were homogenized in 0.1 M Tris-HCL buffer (pH = 7.5) at a ratio of 1:4 (weight:volume), using a Potter-Elvejhem homogenizer at 4°C. The formation of thiobarbituric acid reactive substances (TBARS), as an indicator of lipid peroxidation, in the homogenate was assessed by the method described by Ohkawa et al [23]. In brief, a 3 mL reaction mixture containing 0.1 mL of liver tissue homogenate, 0.2 mL of sodium dodecyl sulfate, 1.0 mL of acetic acid (pH = 3.5, adjusted with 1 M NaOH), 1.5 mL of aqueous solution of thiobarbituric acid (pH = 2.5), and 0.5 mL of a thiobarbituric acid reagent (13.6 g of thiobarbituric acid and 0.35 mL of 3.7 M HCl in 100 mL of 0.2 M sodium hydroxide) were added. The mixture was heated at 100°C for 3 min. After cooling, the spectrophotometric absorbance was measured at 532 nm.
acid (TBA), and 0.7 mL water was prepared. After heating at 95°C for one hour in a hot water bath and cooling, 1 mL of distilled water and 5 mL mixture of n-butanol and pyridine (15:1, v/v) were added. Then, the mixture was shaken vigorously on a vortex mixer and was centrifuged at 3,000 rpm for 10 minutes. The absorbance of the upper organic layer was read at 532 nm.

**Endogenous antioxidant activity assessment**

Superoxide dismutase (SOD) activities of RBC and liver tissues were measured using commercials kits from Randox Laboratories Ltd (London, UK). One unit of SOD was defined as the amount that caused a 50% inhibition rate of reduction of 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT). Activity of SOD was expressed in U/mg. Hb or U/mg. prot. Catalase activity (CAT) was determined using a commercial kit K773-100 from Biovision Inc. (Milpitas, CA, USA). The activity of CAT was expressed as in U/mg. Hb.

**Reduced glutathione (GSH) assay**

GSH levels in plasma were measured using commercials kits from Randox Laboratories Ltd (London, UK). The GSH contents were expressed in mg/g.

**Carbonylated proteins (CP) assay**

1 ml liver tissue homogenate is diluted with 4 ml phosphate-buffered saline (PBS), containing 10 mM sodium phosphate and 0.15 M NaCl (pH = 7.4). And this was centrifuged twice (10 min, 14,000 rpm) in a tabletop microcentrifuge to eliminate all particulate matter. The diluted proteins are precipitated with 0.5 ml cold trichloroacetic acid (TCA, 20% final concentration) and then collected by centrifugation (25000 rpm) for 5 min. 0.5 ml DNPH (dinitrophenylhydrazone 10 mM) in 2 N HCl is added to the protein pellet to give a final protein concentration of 1-1.5 mg/ml. Samples are allowed to stand in the dark room for 1 h with vortexing every 10 min; they are then precipitated with 15% TCA (final concentration) and centrifuged for 5 min. The supernatants are discarded, and the protein pellets are washed three times with 15% TCA, and then was washed three times with 1 ml ethanol/ethyl acetate (1:1, v/v) to remove free DNPH. Samples are then resuspended in 2 ml 6 M guanidine hydrochloride (GdmCl, dissolved in 2 N HCl, pH = 2.3) at 37 °C for 15 min with vortex mixing. Carbonyl contents are determined from the absorbance at 366 nm. The results were expressed in nmol per mg protein.

**Statistical analysis**

Statistical analysis was performed by 2-way ANOVA using GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA) Version 6. To identify the sources of significant main effect, post hoc comparisons (Tukey’s t test) were used. Data were expressed as mean ± standard deviation (SD), and statistical significance was assigned at $p \leq 0.05$.

**RESULTS**

**Macamide and maceaene contents in petroleum ether extract of BM, YM, and PM**

Macamide variations and contents in maca from different origin or with different colors differed vastly. More than 10 analogues of macamides have been identified from the maca extract [24-27]. To quantify macamide contents in maca plant, the first and key step is to ascertain the variations of macamides. Therefore, HPLC-MS was used to scan the extract of BM, YM, and PM. The results showed BM contained seven macamides, YM and PM contained six and five analogues of macamides, respectively. N-benzylalmitamidine was only found in BM extract. All the identity of macamides was further confirmed by comparison of retention time with those of standard compounds. The contents of macamides range from 1.47 to 15.03 ng/g.DW. The YM extract showed the highest total contents of macamides (average 45.85, BM1-BM3; average 57.49, YM1-YM3; average 41.50, PM1-PM3). And the macamide contents in BM extract was in-between. Maceaene in all the nine samples can be detected and their differentiation of contents was negligible among the samples (Table 1).
Table 1. Maceaine and macamide contents of BM, YM, and PM.

<table>
<thead>
<tr>
<th>compounds</th>
<th>BM1 (pg/g. DW)</th>
<th>BM2 (pg/g. DW)</th>
<th>BM3 (pg/g. DW)</th>
<th>YM1 (pg/g. DW)</th>
<th>YM2 (pg/g. DW)</th>
<th>YM3 (pg/g. DW)</th>
<th>PM1 (pg/g. DW)</th>
<th>PM2 (pg/g. DW)</th>
<th>PM3 (pg/g. DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Benzylhexadecanamide</td>
<td>12.3±2.11</td>
<td>12.5±1.33</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>4.38±0.58</td>
<td>5.07±0.65</td>
<td>4.60±0.44</td>
<td></td>
</tr>
<tr>
<td>N-Benzyl-5-oxo-6E,8E-octadecanamide</td>
<td>1.47±0.20</td>
<td>1.52±0.14</td>
<td>1.24±0.14</td>
<td>3.72±0.21</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>N-(3,4-dimethoxy benzyl) hexadecanamide</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>6.94±0.53</td>
<td>7.31±1.38</td>
<td>7.02±0.98</td>
<td>3.68±0.45</td>
<td>3.50±0.45</td>
<td>4.12±0.78</td>
</tr>
<tr>
<td>N-benzyl-tetracosanamide</td>
<td>2.31±0.10</td>
<td>2.24±0.34</td>
<td>1.79±0.23</td>
<td>3.91±0.42</td>
<td>4.35±0.43</td>
<td>3.98±0.23</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Oleamide</td>
<td>3.90±0.62</td>
<td>4.64±0.59</td>
<td>3.82±0.21</td>
<td>12.53±1.78</td>
<td>13.17±1.02</td>
<td>12.54±1.65</td>
<td>6.23±0.78</td>
<td>5.21±0.65</td>
<td>6.03±0.45</td>
</tr>
<tr>
<td>Anandamide</td>
<td>7.92±0.98</td>
<td>8.07±1.23</td>
<td>8.57±1.23</td>
<td>14.87±2.58</td>
<td>15.03±1.69</td>
<td>16.11±1.98</td>
<td>11.53±1.23</td>
<td>12.93±1.65</td>
<td>12.93±0.52</td>
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<tr>
<td>N-benzylpalmitamide</td>
<td>2.22±0.17</td>
<td>2.71±0.11</td>
<td>2.06±0.31</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>N-benzyloleamide</td>
<td>14.70±1.98</td>
<td>13.92±2.37</td>
<td>15.40±1.32</td>
<td>14.20±0.23</td>
<td>14.59±1.78</td>
<td>14.28±2.01</td>
<td>15.63±1.47</td>
<td>15.17±1.02</td>
<td>13.93±1.35</td>
</tr>
<tr>
<td>Maceaine</td>
<td>20.25±2.32</td>
<td>19.67±1.89</td>
<td>21.34±2.78</td>
<td>22.23±2.39</td>
<td>20.73±1.47</td>
<td>20.91±0.87</td>
<td>21.32±2.06</td>
<td>21.56±5.55</td>
<td>20.69±3.27</td>
</tr>
<tr>
<td>Total Macamide</td>
<td>44.90</td>
<td>45.66</td>
<td>47.00</td>
<td>56.17</td>
<td>58.70</td>
<td>57.58</td>
<td>41.45</td>
<td>41.90</td>
<td>41.15</td>
</tr>
</tbody>
</table>

ND: not detected; DW: dried weight. Average retention time of compounds was as followings: N-benzylhexadecanamide 13.6 min; N-benzyl-5-oxo-6E,8E-octadecanamide 16.8 min; N-(3,4-dimethoxy benzyl) hexadecanamide 14.8 min; N-benzyl-tetracosanamide 18.9 min; oleamide 25.8 min; anandamide 20.7 min; N-benzylpalmitamide 29.5 min; N-benzyloleamide 32.0 min and maceaine 34.2 min.

Oral administration of macea extract decreased TBARS and CP contents of liver of DM rat model

TBARS contents of livers were significantly increased after induction of DM in rats. TBARS levels were significantly decreased after 60 d oral administration of BM, YM, and PM extracts but still higher than that in N-CK. The TBARS levels in YM and PM group were decreased to a lower degree when compared with those in BM group. The change of CP of liver was similar to those of TBARS (Figure 2). All the three macea extracts can decrease CP levels in liver. The decreased levels in YM group were more notable than those in BM and PM group. The CP level in YM group was decreased to an even lower than those in N-CK group (Figure 3).

![Figure 1](image1.png)

**Figure 1.** Effect of oral administration of BM, YM, and PM extracts on plasma glucose of DM rat model

![Figure 2](image2.png)

**Figure 2.** Effect of oral administration of BM, YM, and PM extracts on TBRAS contents of liver of DM rat model
Oral administration of PM extract increased SOD and CAT activity of RBC and liver of DM rat model

In the D-CK model, SOD and CAT activity of liver and RBC were significantly decreased compared with those of N-CK model. The activities of SOD and CAT of RBC and SOD of liver tissues were significantly increased after oral administration of BM extract. YM extract can significantly enhance SOD activity of liver. YM extract did not affect the activities of SOD and CAT of RBC and CAT of liver. While the three PM (PM1-PM3) extracts significantly enhanced SOD and CAT activity of both RBC and liver (Table 2).

Oral administration of PM extract enhanced GSH contents in plasma of DM rat model

The GSH contents in plasma of D-CK rat were significantly declined compared with those of N-CK. While, the GSH contents were increased after oral administration of PM extract. Oral administration of BM and YM extracts did not affect GSH contents in plasma on DM rats (Figure 4).
DISCUSSION

Maca has been thought to be a promising plant recently. However, its pharmacological investigation has been focused on increasing fertility ability. Maca quality was different due to its origin and colors. No standard of quality control has been set.

Macamides and maceaine were considered as the unique substance in the plant of maca. Seven, six, and five macamides were identified from the petrol ester extract of black, yellow, and purple maca in the present study, respectively (Table 1). Macamide variations and contents in the maca with the same skin colors was the uniform. The variations and contents of macamides are only dependent on skin colors of maca. By far, the maca was only cultivated in Yunnan province due to the high altitude and unique climatic conditions, in China. Although the samples were collected at random and the specific origin of the plant materials was not confirmed, the maca was ascertained to be from Yunnan Province. It was possible that the variations and contents of macamides were not affected in the maca with the same skin colors since the natural environment for cropping maca was similar. Maceaene was found in all the tested nine samples and the contents differentiation was negligible (Table 1). Whether the minute differentiation of maceaene was caused by sample or cropping environment was not confirmed since no control was supplied in the present study. Macamides belonged to the derivatives of fatty acid, which was known as antioxidant substance. In the present study, we hypothesized that macamides in the petrol ester extract was contributed to antioxidant activity [28,29].

STZ diabetic rats was generally developed into typical diabetic complications [30]. Various proteins, including haemoglobin, albumin, collagen, LDL or crystalline proteins undergo non-enzymatic glycation [31], which may induce oxidative stresses by the formation of oxygen-derived free radicals under diabetic condition [32]. In the present study, the administration of PM YM, and BM extracts all decreased plasma glucose levels in DM rats and prevented STZ-induced oxidative stress (Figure 1). This suggested that maca could ameliorate the oxidative stress caused by hyperglycaemia. The concentrations of lipid peroxides in the tissues are low under physiological conditions. Elevated levels of lipid peroxides in the plasma of diabetic rats was observed [33], which is a free-radical-induced process due to oxidative deterioration of PUFA and resulted in the development of both type I and II diabetes [34]. The most commonly used indicators of lipid peroxidation are TBARS products [35]. The increased lipid peroxidation in the tissues of diabetic animals may be due to the observed increase in the concentration of TBARS in the liver and kidney of diabetic rats [36]. Our results showed that the levels of TBARS in D-CK animals were higher in liver tissue than those of N-CK group, and were restored to normal values after the treatment with maca extract of PM, BM, and YM (Figure 2).

CP is another feature of irreversible oxidative damage, often leading to a loss of protein function, which is considered as a widespread indicator of severe oxidative damage and disease-derived protein dysfunction. STZ-induced oxidative damage in proteins was revealed by the increased content of CP in the plasma and liver tissue [37]. The treatment with three kinds of maca extracts all lowered the contents of CP in liver tissues of DM rats (Figure 3). The decreased levels in PM group were more notable than those in BM and YM group. Oxidative stress in diabetes coexists with a reduction in the antioxidant capacity, which can increase the deleterious effects of the free radicals. SOD protects tissues against oxygen free radicals by catalysing the removal of superoxide radical, converting it into H$_2$O$_2$ and molecular oxygen, which both damage the cell membrane and other biological structures [38]. Catalase is a haem-protein and responsible for the detoxification of significant amounts of H$_2$O$_2$ [39]. Reduced activities of SOD and catalase in the liver and pancreas during diabetes were reported, resulting in a number of deleterious effects due to the accumulation of superoxide radicals and hydrogen peroxide [40]. The activities of SOD and CAT of RBC as well as SOD of liver were significantly increased after oral administration of BM extract. YM extract can only enhance SOD activity in liver. On the other hand, PM extract significantly enhanced SOD and CAT activity of both RBC and liver (Table 2). The DM rats treated with maca extracts showed the reduced TBARS and CP levels, which may be associated with an increased activities of SOD and CAT. GSH is a major intracellular non-protein sulphhydryl compound and is accepted as the most important intracellular hydrophilic antioxidant [41]. Also, GSH acts as a co-substrate for GPx activity and as a co-factor for
many enzymes and stress resistance of many cells is associated with the elevation of GSH levels. A decreased GSH content may predispose the cells to a lower defense against condition of oxidative stress during several degenerative disease conditions including diabetes [42]. The GSH contents were increased only after oral administration of PM extract in the present study (Figure 4). This indicated that DM rats model achieved higher antioxidant defense against STZ-induced oxidative stress.

CONCLUSION

Although the petroleum ether extract of maca with different skin colors showed antioxidant ability to DM rats, no correlation of the contents of macamides with antioxidant ability was observed. Some other compounds may be also attributed to the antioxidant activity. However, as a whole, the PM extract possessed the most potent antioxidant ability among the three species of maca.

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