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<th>著者</th>
<th>ヨイチ Shimoda, ヒョクトウ Han, キヨカズ Kawada, サムウ Smaoui, ヒロコーボ Isoda</th>
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Research Article

Metabolomics Analysis of *Cistus monspeliensis* Leaf Extract on Energy Metabolism Activation in Human Intestinal Cells

Yoichi Shimoda,1 Junkyu Han,1, 2 Kiyokazu Kawada,1, 2 Abderrazak Smaoui, 3 and Hiroko Isoda1, 2

1 Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan
2 Alliance for Research on North Africa, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan
3 Laboratory of Extremophile Plants, Center of Biotechnology of Borj-Cedria, 2050 Hammam-Lif, Tunisia

Correspondence should be addressed to Hiroko Isoda, isoda.hiroko.ga@u.tsukuba.ac.jp

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Energy metabolism is a very important process to improve and maintain health from the point of view of physiology. It is well known that the intracellular ATP production is contributed to energy metabolism in cells. *Cistus monspeliensis* is widely used as tea, spices, and medical herb; however, it has not been focusing on the activation of energy metabolism. In this study, *C. monspeliensis* was investigated as the food resources by activation of energy metabolism in human intestinal epithelial cells. *C. monspeliensis* extract showed high antioxidant ability. In addition, the promotion of metabolites of glycolysis and TCA cycle was induced by *C. monspeliensis* treatment. These results suggest that *C. monspeliensis* extract has an ability to enhance the energy metabolism in human intestinal cells.

1. Introduction

A lot of natural herbs distributed around the Mediterranean Sea have been traditionally used by local people [1]. Most of the studies on plants to grow for Mediterranean climate have been investigated mainly in tolerance for the drying and relationship with environmental stress. However, the physiological function of natural herb is still poorly understood though many species grow all over the place. In an area of North Africa, the several species of natural herb were employed in traditional medicine as active against *Helicobacter pylori*, oxidative stress, hypertension, and hypoglycaemic [2–4]. *Cistus monspeliensis* is a perennial plant which is widely distributed from South Europe to North Africa. The genus *Cistus* is popular on tea and spice but not utilized for the antiaging effects. *C. monspeliensis* has been reported to have an antioxidation, antibacterial, and anti-inflammatory effects [5].

In the small intestine, goblet cells secrete mucus that forms a coating over the epithelial layer. The main function of epithelial layer includes absorption of food compounds [6]. The ingested capsaicin was carried into the intestinal epithelium, which is in contact with a high concentration of food ingredients [7, 8]. Considering the relevance of the food components and the effects of the oral route for human exposure, we have investigated the effect of capsaicin on the energy metabolism of intestine, by using Caco-2 cell line, a well-known *in vitro* model of intestinal epithelium [6].

The intracellular ATP accumulation is important for optimal integrity of the mucosa and has been suggested to play a specific role in the regulation of absorption and barrier functions [9, 10]. From these reports, it was supposed that the intracellular ATP accumulation contributes to the homeostasis of Caco-2 cells, because the regulation of absorption and barrier functions are necessary for Caco-2 cells differentiation [11].

In this study, the energy metabolism underlying the effect of *C. monspeliensis* extract on intestinal epithelium was clarified by performing the capillary electrophoresis time-of-flight mass spectrometry (CE-ToF/MS) analysis. Moreover, we performed real-time PCR to quantitate mRNA using the primers, related with ATP production, and the luciferase
assay was performed to measure intracellular ATP production in the intestinal epithelium. This is the first report that the extract of *C. monspeliensis* was induced by the activation of energy metabolism in human intestinal epithelial cells.

2. Materials and Methods

2.1. Cell Culture. Human intestinal epithelial Caco-2 cells were obtained from American Type Culture Collection (Manassas, VA, USA) and were used between passages 10 to 30. The cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Sigma, St. Louis, MO) supplemented with 10% (v/v) fetal bovine serum (Hyclone Co., Ltd.), 1% (v/v) penicillin-streptomycin (Lonza, Walkersville, MD USA), and 1% (v/v) nonessential amino acids (Cosmo Bio Co., Ltd., Tokyo, Japan) and incubated in an atmosphere of 5% CO₂ at 37°C.

2.2. Plant Materials and Extracts Preparation. 23 plants were collected between June and July in 2008 from Jendouba, Kairouan, and Kasserine areas in Tunisia and air-dried in the shade at room temperature and ground to powder with an electrical blender and stored at room temperature. Each plant sample (1 g) was extracted with 10 mL of distilled water at 105°C for 15 minutes or with 10 mL of 70% (w/v) ethanol at room temperature for 1 week. The extract water was filtered and sterilized using 0.22 μm membrane filter and stored at −80°C.

2.3. DPPH Assay. The antioxidant effect of plants was determined by DPPH (1,1-diphenyl-2-picrylhydrazyl) assay performed. DPPH (2 mg) was dissolved in ethanol (12.76 mL). The ethanol solution (5 mL), 400 mM 2-morpholinoethanesulfonic acid monohydrate (MES) solution (12.76 mL), and MilliQ water (4.5 mL) were mixed in one tube. Furthermore, plants extract (10 μL) and the mixing solution (190 μL) were added in 96-well plates and incubated for 10 min at room temperature. The absorbance was spectrophotometrically determined at 520 nm using a multidetection microplate reader (Powerscan HT, Dainippon Pharmaceutical, USA).

2.4. Total RNA Isolation and Real-Time PCR. After incubating seeded plates for 24 h, total RNA was purified using the ISOGEN kit (Nippon Gene Co., Ltd., Japan.) Total RNA was quantified by measurement at 260 nm with a UV spectrophotometer and was also measured at 280 nm to assess purity. Only RNA with a 260/280 ratio higher than 1.8 was used for the real-time PCR. Template cDNA was obtained from total RNA using the SuperScript reverse transcriptase system (Invitrogen). Briefly, RNA was denatured at 65°C for 5 min and incubated with 1 mL oligo (dT) 12–15 primers and chilled at 4°C. After adding SuperScript II reverse transcriptase (200 units), the reaction mix was incubated at 42°C for 60 min, then 10 min at 70°C. For the quantification of mRNA, nested primers were designed using Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Quantitative PCR reactions were performed in a MiniOpticon instrument (Bio-Rad, USA). Briefly, the RT mix (2 mL) was used as template for the real-time PCR mix containing 0.5 mM forward and reverse nested primers (2 μL each) and 2 × SYBR Green supermix (10 μL). The primers used were checked using the BLASTn program of the GeneBank; their sequence (TPI: forward: 5’-CTTGCTGAGATGGAAGG-3’, reverse: 5’-CAGTGAAGGCAGACAAACC-3’; PGM: forward: 5’-CGGAGAATACTCATCCAGAG-3’, reverse: 5’-TGTCAGATGATCCACCAA-3’; ATP synthase: forward: 5’-CTGGAGGACCTGTGGATGCT-3’, reverse: 5’-TGGGGTTTTCTCGATGACTTC-3’) was based on the known sequences in the cording region of the human genes. The amplification conditions were 3 min at 95°C, 10 sec at 95°C, 30 sec at 62°C, and 30 sec 72°C for 41 cycles. At the end of the reaction, a melting curve analysis was carried out to check for the presence of primer dimers.

2.5. Metabolomics Analysis. After incubating seeded plates for 24 h, the extract was added at 0.1% (w/v) concentration and the cells were incubated for 12 h. After treatment, 5% (w/v) mannitol solution was added at 10 mL and was removed. Once again, 5% (w/v) mannitol solution was added at 2.0 mL and was removed. Cells were scraped in 1.3 mL of methanol which includes 10 μM each of 2 internal standards (ISC1 and ISA1), and methanol including scraped cells was transferred at 1.0 mL to centrifuge tube. 1 mL of chloroform and 400 μL of MilliQ water were added to the solution and then thoroughly mixed. Followed by centrifugation at 2,300 g for 5 min at 4°C, the 250 μL of water layer was removed and centrifugally filtered through a 5-kDa-cutoff filter (Millipore, USA) to remove proteins. The filtrate was desiccated and dissolved in 20 μL of MilliQ water prior to injection. The capillary electrophoresis time-of-flight mass spectrometry (CE-ToF/MS) experiments were performed using an Agilent CE-ToF/MS system (Agilent Technologies Co.) unit 5. Separations were carried out on a fused silica capillary (50 μm i.d. × 80 cm total length). CE-ToF/MS conditions for anionic metabolites were followed. Run buffer: anion buffer solution (p/n: H3302-1021), rinse buffer: anion buffer solution (p/n: H3302-1022), sample injection: pressure injection 50 mbar, 25 sec, CE voltage: positive, 30 kV, MS ionization: ESI negative, MS capillary voltage: 3,500 V, MS scan range: n/z 50–1,000, sheath liquid (p/n: H3312-1020) [12].

2.6. ATP Measurement. ATP was assessed by firefly bioluminescence using the luminescence luciferase assay kit (TOYO Ink, Tokyo, Japan). Caco-2 cells were plated in 96-well plates at 1.0 × 10⁵ cells/mL in 100 μL. After 24 h incubation, the extract was added at 0.1, 0.01, and 0.001% (w/v) concentration, and the cells were incubated for 3, 6, and 12 h. After treatment, 100 μL of luciferin-luciferase solution was added and stirred for 3 min using a microplate genie, 100 V (Scientific industries, Inc., USA). The luminescence was determined using a multidetection microplate reader (Powerscan HT, Dainippon Pharmaceutical, USA).

2.7. MTT Assay. The viability of cells was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
investigate the effect of C. monspeliensis extract on the actin-
duction by Caco-2 Cells. In the results of real-time PCR and
mRNA expression involved ATP production. We deter-
mined ATP production of Caco-2 compared with nontreated cells.
As a result, we found that C. monspeliensis extract increased ATP production.

3. Results

3.1. Effect of Antioxidant of 23 Plants Extracts. DPPH radical
is well known as a stable organic free radical which has been
used for estimation of the antioxidant capacity. DPPH
radical is changed to nonradical to react antioxidants.
We determined the antioxidation of 23 plants grown around
the Mediterranean Sea. Especially, eighteen plants had higher
antioxidation more than 75% (Table 1). We selected C. mon-
speliensis in twenty three plants that had high antioxidation
to assess ATP production because this plant was used to
traditional foods.

3.2. Effect of C. monspeliensis Extract on the Expression of
TPI, PGM, and ATP Synthase mRNA by Caco-2 Cells. To
investigate the effect of C. monspeliensis extract on the activ-
ation of energy metabolism in human intestinal epithelium
(Figure 1), real-time PCR was employed to evaluate the
mRNA expression of genes, related with glycolysis and
TCA cycle. In this experiment, we used the primers of
triosephosphate isomerase (TPI), phosphoglycerate mutase
(PGM), and ATP synthase that have a deep relationship
with intracellular ATP production and relatively higher
expression in Caco-2 cells. Especially, to treat 0.1% (w/v)
C. monspeliensis for 6 h, mRNA expression levels of TPI,
PGM, and ATP synthase were upregulated by 170%, 161%,
and 310%, respectively. PGM is an enzyme that catalyzes
the internal transfer of a phosphate group from C-3 to
C-2 which results in the conversion of 3-phosphoglycerate
to 2-phosphoglycerate through a 2, 3-bisphosphoglycerate
intermediate. TPI is an enzyme that catalyzes the reversible
interconversion of the triose phosphate isomers, dihydroxy-
acetone phosphate, and D-glyceraldehyde 3-phosphate. TPI
plays an important role in glycolysis and is essential for
efficient energy production. ATP synthase is an important
enzyme that creates energy for the cell to use through
the synthesis of ATP in mitochondria. In Figure 1, mRNA
expression of TPI and PGM, glycolytic enzymes, increased
to treat with C. monspeliensis extract. Also, ATP synthase,
electron transport chain enzyme, was increased.

3.3. Effect of C. monspeliensis Extract on the Metabolomics
by Caco-2 Cells. As a result of the metabolomics analysis,
C. monspeliensis extraction increased production of each
metabolite in glycolysis and TCA cycle (Table 2). Glycolysis
is a pathway of ATP production in anaerobic. In this result,
three metabolites on rate-limiting step in glycolysis (glucose
6-phosphate, fructose 1,6-diphosphate, and pyruvic acid)
were increased by C. monspeliensis extraction. Especially,
pyruvic acid was the most increased in glycolytic metabolites.
The production of pyruvic acid is related to promote
function of ATPase that involved ATP synthesis [13]. ATP is
not produced in TCA cycle; however, a lot of ATP is produced
in electron transport chain. Antising on promoting energy
metabolism is expected to promote function of glycolytic
and electron transport chain enzymes that are pathways of ATP
production.

3.4. Effect of C. monspeliensis on the Intracellular ATP Pro-
duction by Caco-2 Cells. In the results of real-time PCR and
metabolomics, C. monspeliensis was upregulated metabolite
and mRNA expression involved ATP production. We deter-
mined ATP production of C. monspeliensis extract using
the luminescence luciferase assay kit (Figure 2). 0.1, 0.01,
and 0.001% (w/v) of C. monspeliensis extraction increased
ATP production on Caco-2 compared with nontreated cells.
Especially, to treat 0.01% (w/v) of C. monspeliensis for 3 h,
ATP production was upregulated by 172%. As a result, we

Table 1: Effect of antioxidant of 23 plants extracts. The radical
2,2-diphenyl-1-picrylhydrazyl (DPPH) was used for measuring the
antioxidants. The hot water extract and the ethanol extract were
diluted to 10 or 100 times by each solution. The values of
antioxidant were calculated by the following formula: antioxidant
(%) = 1 − (Absorbance520 (Sample)/Absorbance520 (Control)) ×
100.

<table>
<thead>
<tr>
<th>Name of plants</th>
<th>EtOH extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1% (w/v)</td>
</tr>
<tr>
<td>Ajuga iva</td>
<td>14</td>
</tr>
<tr>
<td>Artemisia campestris</td>
<td>38</td>
</tr>
<tr>
<td>Artemisia herba-alba</td>
<td>2</td>
</tr>
<tr>
<td>Cistus monspeliensis</td>
<td>24</td>
</tr>
<tr>
<td>Cyperus longus</td>
<td>10</td>
</tr>
<tr>
<td>Daphne gnidium</td>
<td>5</td>
</tr>
<tr>
<td>Erica multiflora</td>
<td>24</td>
</tr>
<tr>
<td>Globularia alypum</td>
<td>18</td>
</tr>
<tr>
<td>Lavandula nobilis</td>
<td>10</td>
</tr>
<tr>
<td>Lavandula angustifolia</td>
<td>13</td>
</tr>
<tr>
<td>Lavandula officinalis</td>
<td>2</td>
</tr>
<tr>
<td>Lavandula stoechas</td>
<td>3</td>
</tr>
<tr>
<td>Marrubium vulgare</td>
<td>8</td>
</tr>
<tr>
<td>Mentha rotundifolia</td>
<td>8</td>
</tr>
<tr>
<td>Mentha viridis</td>
<td>12</td>
</tr>
<tr>
<td>Origanum majorana</td>
<td>13</td>
</tr>
<tr>
<td>Phillaria angustifolia</td>
<td>15</td>
</tr>
<tr>
<td>Pinus halepensis</td>
<td>11</td>
</tr>
<tr>
<td>Rhamnus lycioides</td>
<td>10</td>
</tr>
<tr>
<td>Teucrium polium</td>
<td>10</td>
</tr>
<tr>
<td>Thymus capitatus</td>
<td>13</td>
</tr>
<tr>
<td>Vitex agnus</td>
<td>13</td>
</tr>
<tr>
<td>Ziziphus lotus</td>
<td>15</td>
</tr>
</tbody>
</table>
Figure 1: Effect of *C. monspeliensis* extract on the expression of TPI (a), PGM (b), and ATP synthase (c) mRNA by Caco-2 cells. β-actin was used as a house-keeping gene. The mRNA expression of TPI, PGM, and ATP synthase was normalized by β-actin mRNA expression. Each bar represents the mean ± SD (n = 3). *P < 0.05, **P < 0.01 compared to the control as determined by the t-test.

Table 2: Effect of *C. monspeliensis* extract on the metabolomics by Caco-2 cells. Quantified levels of metabolites involved in glycolysis pathway were determined. Caco-2 cells were treated with 0.1% (w/v) *C. monspeliensis* extract for 12 h. The ratio of quantified levels of metabolites was calculated by Caco-2 cells treated or nontreated with *C. monspeliensis* extract.

<table>
<thead>
<tr>
<th>Metabolite</th>
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<tr>
<td><strong>Glycolysis</strong></td>
<td></td>
</tr>
<tr>
<td>Glucose 6-phosphate</td>
<td>1.7</td>
</tr>
<tr>
<td>Fructose 6-phosphate</td>
<td>1.6</td>
</tr>
<tr>
<td>Fructose 1,6-diphosphate</td>
<td>1.5</td>
</tr>
<tr>
<td>Dihydroxyacetone phosphate</td>
<td>2.1</td>
</tr>
<tr>
<td>3-Phosphoglyceric acid</td>
<td>1.0</td>
</tr>
<tr>
<td>Phosphoenolpyruvic acid</td>
<td>0.8</td>
</tr>
<tr>
<td>Pyruvic acid</td>
<td>4.2</td>
</tr>
<tr>
<td><strong>TCA cycle</strong></td>
<td></td>
</tr>
<tr>
<td>Acetyl CoA,divalent</td>
<td>0.7</td>
</tr>
<tr>
<td>Citric acid</td>
<td>1.5</td>
</tr>
<tr>
<td>Cis-aconitic acid</td>
<td>1.4</td>
</tr>
<tr>
<td>Isocitric acid</td>
<td>1.0</td>
</tr>
<tr>
<td>2-Oxoglutaric acid</td>
<td>1.6</td>
</tr>
<tr>
<td>Succinic acid</td>
<td>0.8</td>
</tr>
<tr>
<td>Fumaric acid</td>
<td>1.1</td>
</tr>
<tr>
<td>Malic acid</td>
<td>1.0</td>
</tr>
</tbody>
</table>

considered that *C. monspeliensis* had effect of ATP production and to promote energy metabolism on Caco-2.

3.5. Effect of *C. monspeliensis* on the Cell Viability by Caco-2 Cells. We determined cell proliferation on Caco-2 using MTT assay. From the result of MTT assay, 0.01 and 0.001% (w/v) of *C. monspeliensis* extraction were not changed cell proliferation on Caco-2 cells, whereas 0.1% (w/v) of *C. monspeliensis* extraction induced the increase of cell proliferation on Caco-2 cells compared with nontreated cells. As a result, we considered that 0.1 to 0.001% (w/v) *C. monspeliensis* extract had not toxic effect for Caco-2 cells.

4. Discussion

ATP is a multifunctional nucleotide that is the most important as a "molecular currency" of intracellular energy transfer. In this role, ATP transports chemical energy within cells for metabolism. The glycolytic and TCA cyclic enzymes played an important role in the intracellular ATP production [14, 15]. TPI enzyme is essential for energy production, allowing two molecules of glyceraldehyde 3-phosphate to be produced for every glucose molecule, thereby doubling the energy yield. PGM enzyme catalyzes 1,3-bisphosphoglycerate and plays an important role downstream of glycolysis. The activity of TPI and PGM was not influenced by age or caloric restriction [16], while the other glycolytic enzymes were influenced. Furthermore, the activity and expression of these two enzymes, however, are
myricetin, and flavan-3-ols [28]. For example, quercetin has
lar ATP. From these results, we suggest that C. monspeliensis
species of extract can be used as antiaging e
monspeliensis extract enhanced the production of intracellu-
ATP production, in human intestinal epithelial cells. And
duced the expression of enzymes, related with intracellular
production can make use of antiaging e
functional foods for antiaging.
Also, it is known that function of SOD decreased [27]. From
mal recessive inheritance [19] and neurodegeneration [20].
known to cause a severe multisystemic disease with autoso-
glyceraldehydes 3-phosphate (GAP). Its deficiency has been
TPI isomerizes dihydroxy acetone phosphate (DHAP) to
logical energy conversion. ATP synthase uses the proton mo-
motor in response to the cellular energy demand has
thase seems to be a central physiological phenomenon which
muscle, fibroblasts, and brain. Regulation of the ATP-syn-
been demonstrated in several species like rat, dog, and hu-
and different types of tissue like heart muscle, skeletal
muscle, fibroblasts, and brain. Regulation of the ATP-syn-
has been demonstrated in several species like rat, dog, and hu-
ments that accompany Alzheimer’s disease [29]. Moreover,
ence of intestinal absorption [30, 31]. We expect that C. monspeliensis extract can contribute to prevent several diseases like senescence, Alzheimer’s disease, cardioclerosis, senoccardia, cancer, and so forth and recover the function of impairment intestinal absorption.
0.001, 0.01% (w/v) of C. monspeliensis increased ATP produc-
tion (Figure 2) and, however, did not changed cell
viability as compared with low-concentration sample and,
regulated cell viability in MTT assay. From these results, we considered that ATP was consumed for
cell proliferation in 0.1% (w/v) of C. monspeliensis, and
antioxidation and has a function to improve memory impair-
ment that accompany Alzheimer’s disease [29]. Moreover, it is known that an aging is caused for decline of intestinal absorption [30, 31]. We expect that C. monspeliensis extract can contribute to prevent several diseases like senescence, Alzheimer’s disease, cardioclerosis, senoccardia, cancer, and so forth and recover the function of impairment intestinal absorption.
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duction as compared with low-concentration sample and,
regulated cell viability in MTT assay. From these results, we considered that ATP was consumed for
cell proliferation in 0.1% (w/v) of C. monspeliensis, and
ATP accumulation a little decreased as compared with low concentration (0.01 and 0.001% (w/v)). It was reported that the ATP accumulation was contributed to the proliferation and homeostasis on Caco-2 cells [10]. We expect that C. monspeliensis extract can induce the ATP accumulation and then the activation of proliferation and homeostasis on Caco-2 cells.

We considered that isolation of active component from C. monspeliensis extract should be fulfilled and further in vivo studies should focus on the confirmation of activation and safety of C. monspeliensis extract.

5. Conclusion

Our findings indicate that C. monspeliensis extract had high antioxidant ability. In addition, we show that C. monspeliensis extract has the function of promoting energy metabolism pathways, including glycolysis, TCA cycle, and electron transport chain, in human intestinal epithelial cells. Also, C. monspeliensis extract enhanced the production of intracellular ATP on intestinal epithelium. From these results, C. monspeliensis extract has an ability to enhance the energy metabolism. Further studies are in progress to elucidate the effect of C. monspeliensis on antiaging.

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References


