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Study on glucose transport in muscle cells by extracts from Mitragyna speciosa (Korth) and mitragynine

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Study on glucose transport in muscle cells by extracts from *Mitragyna speciosa* (Korth) and mitragynine

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The leaves of *Mitragyna speciosa* Korth (Rubiaceae) have been used in folk medicine for its unique medicinal properties. This study examined the water, methanolic and crude alkaloidal extracts from *M. speciosa* leaves and its major constituent mitragynine for the enhancement of glucose transport. Cellular uptake of radioactive 2-deoxyglucose was determined in rat L8 myotubes. Involving signalling pathway was determined with the specific inhibitors. Cell cytotoxicity was monitored by lactate dehydrogenase assay. Protein levels of glucose transporters (GLUTs) were measured by Western blotting. The results show that test samples significantly increased the rate of glucose uptake. The uptake was associated with increase in GLUT1 protein content. Co-incubation with insulin had no additional effect, but the cellular uptake was decreased by wortmannin and SB 203580, specific inhibitors of phosphatidylinositol 3-kinase (PI3K) and p38 mitogen-activated protein kinase (p38 MAPK), respectively. It is concluded that the increased glucose transport activity of *M. speciosa* is associated with increases in activities of the key enzymes dependent to the insulin-stimulated glucose transport for its acute action, and increases in the GLUT1 content for its long-term effect. This study demonstrated the effect of *M. speciosa* in stimulating glucose transport in muscle cells, implicating the folkloric use of *M. speciosa* leaves for treating diabetes.

**Keywords:** *Mitragyna speciosa*; mitragynine; glucose uptake; L8 muscle cells

1. Introduction

*Mitragyna speciosa* Korth or Kratom (Thai name) is a tree native to Southeast Asia. The leaves of Kratom have been used in folk medicine as a stimulant (at low doses) to combat fatigue and enhance tolerance to hard work under the scorching sun, sedative (at high doses), pain killer, and treatment for opiate addiction and weaning off morphine addiction (Jansen & Prast, 1998). The aqueous extract of Kratom significantly inhibits...
ethanol withdrawal-induced behaviours including rearing, displacement and head weaving
in mice (Kumarnsit, Keawpradub, & Nuankaew, 2007). The methanolic Kratom extract
exhibits an antidiarrheal effect on rat gastrointestinal tract (Chitrtrakarn, Sawangjaroen,
Prasettho, Janchawee, & Keawpradub, 2008). Additionally, Kratom has been traditionally
used as a medicine to treat diabetes, especially in Thailand, without much scientific
evidence of its utility.

The major constituent mitragynine, and some other indole alkaloids in the leaves are
responsible for Kratom’s pharmacological activities. Mitragynine has an antinociceptive
action through the supraspinal opioid receptors, and its action is dominantly mediated by
µ- and δ-receptor subtypes in in vivo and in vitro studies (Matsumoto et al., 1996a, 1996b;
Thongpraditchote et al., 1998). 7-Hydroxymitragynine is a novel opioid agonist and has
potent analgesic activity when orally administered (Matsumoto et al., 2004). Chemistry
and pharmacology of analgesic indole alkaloids from Kratom have been intensively
reviewed (Takayama, 2004).

In this work, we evaluated the hypoglycemic effect of the crude watery,
methanolic and alkaloidal extracts of Kratom leaves, and mitragynine by assessing
glucose transport into muscle cells in culture. The mechanism of action at cellular
level was presented. This is the first report demonstrating the anti-diabetic activity of
M. speciosa.

2. Materials and methods
2.1. Materials
L8 cells were purchased from American Type Culture Collection (Rockville, MD).
Cell culture medium and supplements were from Life Technologies, Inc. (Gaithersburg,
MD). Insulin, phloretin, SB 203580, wortmannin, 2-deoxyglucose, protease inhibitor
cocktail and standard chemicals were from Sigma (St. Louis, MO). 2-Deoxy-D-[3H]
glucose (2-dG) was purchased from Amersham Biosciences (Piscataway, NJ). CytoTox 96
non-radioactive cytotoxicity assay was from Promega (Madison, WI). Anti-GLUT1
antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). A monoclonal
antibody to GLUT4 was from Biogenesis (Brentwood, NH). Electrophoresis and protein
assay reagents were from Bio-Rad (Hercules, CA).

2.2. Sample preparation
2.2.1. Plant material
The leaves of M. speciosa Korth (Rubiaceae) were collected from natural sources in
Songkhla province, southern Thailand. Authentication of plant material was carried out at
the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of
Pharmaceutical Sciences, Prince of Songkla University, where the herbarium vouchers
(PCOG/MS 001-002) have been deposited. The leaves (2 kg) were dried at 45–50°C and
grounded, to give leaf powder (395 g).

2.2.2. Water extraction
Five grams of the leaf powder were extracted with 200 mL of water at 70°C for 30 min.
The extract was filtered through gauze cloth, centrifuged at 5000 × g for 20 min, and then
lyophilised (watery extract, WE). WE were re-dissolved, filtered-sterile and subsequently employed in the glucose uptake assay.

2.2.3. Methanol extraction
Three hundred grams of the leaf powder were macerated with 3 L of methanol for 2 days (repeated 3 times). The methanol filtrates were combined and evaporated under reduced pressure to give the methanol extract (ME) as a syrupy mass (118 g).

2.2.4. Crude alkaloid extraction
The methanol extract (100 g) was dissolved in 10% acetic acid solution, well shaken and left to stand overnight. The acidic filtrate was washed with petroleum ether, made alkali (pH 9) with 25% ammonia solution and extracted with chloroform. The combined chloroform extract was washed with distilled water, dried over anhydrous sodium sulphate and evaporated to yield dry crude alkaloid extract (AE, 3.23 g).

2.2.5. Isolation of mitragynine
The crude alkaloid extract (1.5 g) was subjected to silica gel column chromatography, eluted with 5% methanol in chloroform to obtain a major alkaloid as amorphous powder (0.74 g), which appeared as a single spot on TLC analysis (4 solvent systems). It was found to be a pure compound according to spectroscopic analysis of MS (Thermofinnigan MAT 95 XL mass spectrometer; EIMS with direct insert probe), $^1$H-NMR and $^{13}$C-NMR (Varian Unity Inova 500 NMR spectrometer) spectra. Comparing the obtained spectral data with the published assignments (Houghton, Latiff, & Said 1991; Shellard, Houghton, & Resha, 1978), it was identified as mitragynine. In this study, mitragynine was accounted for ~49.3, 1.6 and 0.63% of the crude alkaloid extract, methanol extract and leaf powder, respectively.

2.3. Cell culture and glucose transport measurements
Monolayers of L8 muscle cell were grown at 37°C in 24-well or 100 mm culture dishes in Dulbecco’s modified Eagle’s medium (DMEM, low glucose), containing 10% foetal bovine serum (FBS), penicillin (100 units mL$^{-1}$) and streptomycin (100 µg mL$^{-1}$) in incubators equilibrated with 5% CO$_2$. At 95% confluence (day 0) and thereafter, differentiation was induced and maintained in the low serum (2% horse serum, HS) containing medium. Cells were used to investigate the effects of Kratom extracts and mitragynine on 2-dG transport between days 7 and 9 at which ~80–90% of myotubes were formed. The cells were stimulated in HEPES-buffered saline, pH 7.4 (HBS) solution with 2% HS, 15 mM glucose with different concentrations of test agent for 1, 6 or 24 h. Some cells were pre-incubated for 30 min with wortmannin or SB 203580. Some cells were deprived of serum in the medium containing 25 mM glucose, 0.1% BSA for 5 h (Klip, Li, & Logan, 1984) before being exposed to insulin (100 nM), test agent or both. Control cells were also performed in the absence of test sample for the same incubation periods. At the end of the incubation, the test media were washed out with HBS followed by incubation in HBS-2-dG solution (1 µCi mL$^{-1}$, 0.01 µM unlabelled substance) for 10 min in the presence or absence of insulin. The radioactivity associated with the cells was determined by
cell lysis in 0.05N NaOH, and then subjected to liquid scintillation counting. Non-specific uptake was determined in parallel wells containing 10 mM phloretin. The protein content was determined by the method of Bradford (1976), and the specific uptake is expressed in femtomole per milligram protein or percent of control as the mean ± SD from n independent experiments, each assayed in triplicate.

2.4. Western blot analysis of glucose transporters

Whole cell lysate preparation was carried out as described earlier (Purintrapiban, Suttajit, & Forsberg, 2006). In brief, cells were treated with 20 strokes in homogenising buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EGTA, 0.1 mM MgCl₂, protease inhibitors). Cell lysates were centrifuged at 200 × g for 5 min. The supernatant were collected for protein analysis. Protein samples (150 μg) were separated in 10% SDS-PAGE, transferred to PVDF membrane, and probed with antibodies against GLUT4 (1:250), GLUT1 (1:700) and α-actin (1:700). Colorimetric method of alkaline phosphatase detection system was employed. The immunoblots were quantified using scanning densitometer.

2.5. Statistical analysis

All data are expressed as means ± SD. Statistical analyses were performed by a one-way analysis of variance followed by Scheffe tests. The level of p < 0.05 was considered significant.

3. Results

To assess the role of Kratom in glucose transport, we incubated L8 myotubes with different doses of the test agents, and measured the rate of 2-dG uptake into the cells. Figure 1 shows the increased 2-dG uptake activity elicited by 1 h exposure to ME, with
maximal effect by 147% of the basal level at 25 \mu g \text{mL}^{-1}. Mitragynine increased the marked rate of 2-dG uptake at a concentration as low as 40 \mu g \text{mL}^{-1} and the maximum stimulation (141%) was obtained at about 60 \mu g \text{mL}^{-1.} As far as AE is concerned, its stimulation was slight (128%), but significant. At higher levels than the optimum concentrations or in prolonged incubation, the rate was decreased due to cytotoxic effect. Figure 2 shows that WE activated 2-dG transport in a dose- and time-dependent manner. After 1 h treatment, 0.6 mg \text{mL}^{-1} WE was required to significantly activate the 2-dG uptake activity, whereas after 6 h exposure, 0.4 mg \text{mL}^{-1} was sufficient to markedly activate the glucose transport. An increment of the transport activity about 1.26 \pm 0.12 and 2.24 \pm 0.27 fmol/mg protein by 0.1 and 0.2 mg/ml WE, respectively was achieved in 24 h treatment (data not shown). In the subsequent studies, WE was chosen as the test stimulant for its low-toxic effect and high-induction level.

Since WE exhibited an acute response similar to that elicited by insulin, we compared the transport activity of both agents. The effect of WE was not diminished in serum-starved cells under the test condition that was necessary for detecting insulin action (Klip et al., 1984). At its maximum concentration (0.6 mg mL^{-1}), WE increased the rate of 2-dG uptake by 0.674 \pm 0.09 fmol mg^{-1} protein (Figure 3). In the experiment, insulin, a biological activator of glucose transport, increased the rate by 0.545 \pm 0.06 fmol mg^{-1} protein. When WE and insulin were added together, the resulting effect has increased 2-dG uptake, but it was not significant to either the effect of insulin or WE on its own. This was also true on the low level (0.2 mg mL^{-1}) of WE. In order to investigate the association of insulin signalling to WE, cells were pre-incubated for 30 min with 100 nM wortmannin or 10 \mu M SB 203580, then treated with WE for 1 h before assaying the glucose transport. Table 1 presents the averaged results of SB 203580 treatment, showing a significant decrease in the effect of WE on 2-dG uptake induction by 28.56% due to the activation of the transporter intrinsic activity, whereas wortmannin that inhibits the transporter translocation process results in a 15.40% reduction. Finally, we investigated whether

Figure 2. Time- and dose-dependence of the water extract of Mitragyna speciosa leaves (WE) on glucose transport in L8 myotubes. L8 cells were incubated with increasing doses of WE for 1 and 6 h prior to 10 min determination of 2-deoxyglucose uptake. Data shown are means of three independent experiments within which each point was assayed in triplicate. *p < 0.05; t, toxic effect.
WE involved in the upregulation of GLUT1 and GLUT4 protein. Figure 4 shows that exposure of the cells to 0.6 mg mL\(^{-1}\) WE for 1 and 6 h did not change the total content of GLUT4, determined by Western blotting of the total cell lysate. The content of GLUT1 was not affected by the 1 h treatment. In contrast, the amount of GLUT1 protein following 6 h exposure was significantly increased \((p < 0.05)\).

4. Discussion
The aim of this study was to determine the hypoglycemic effect and mechanisms by which \textit{M. speciosa} (Kratom) leaf mediates the anti-diabetic response in human subjects.

Table 1. Effect of wortmannin and SB 203580 preincubation on WE-induced 2-deoxyglucose uptake in L8 myotubes.

<table>
<thead>
<tr>
<th>Condition</th>
<th>2-Deoxyglucose uptake (% of control)</th>
<th>Relative to basal</th>
<th>Relative to WE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal control</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Wortmannin</td>
<td>83.82 ± 2.72</td>
<td>16.18</td>
<td></td>
</tr>
<tr>
<td>SB 203580</td>
<td>77.81 ± 3.96</td>
<td>22.19</td>
<td></td>
</tr>
<tr>
<td>WE</td>
<td>192.76 ± 6.34</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>WE + wortmannin</td>
<td>163.07 ± 9.02*</td>
<td>15.40</td>
<td></td>
</tr>
<tr>
<td>WE + SB 203580</td>
<td>137.71 ± 8.46*</td>
<td>28.56</td>
<td></td>
</tr>
</tbody>
</table>

Notes: L8 myotubes were preincubated with either 100 nM wortmannin or 10 μM SB 203580 for 30 min, washed, treated with or without 0.6 mg mL\(^{-1}\) WE for 1 h. Cells were rinsed then assayed for 2-dG uptake. Results are expressed relative to basal or WE values and represent mean ± SD of four independent experiments.

*\(p < 0.05\) compared with WE.
To accomplish this, we used a cell culture model to study the modulation of the glucose transport system of L8 muscle cells. The results in this study have clearly demonstrated that the water extract of Kratom can elicit significant time- and dose-dependent increases in 2-dG uptake in L8 muscle cells in which activation of insulin-mediated signalling by the WE leads to the effects on glucose transport as resulted under the test conditions we employed. Redistribution of GLUT4 from an intracellular pool to the plasma membrane, as a result of PI3K activation, the rate-limiting step in insulin action that can be blocked by wortmannin, an inhibitor of insulin signalling (Okada, Kawano, Sakakibara, Hazeki, & Ui, 1994; Somwar, Sumitani, Taha, Sweeney, & Klip, 1998), augments the glucose transport. In addition, wortmannin reversed the effect of WE in the cells in which GLUT1 protein was down-regulated by serum deprivation, hence the transport system being operated mostly relies on GLUT4 protein (Klip et al., 1984; Purintrapiban & Ratanachàiayavong, 2003). Increased p38 MAPK activation also serves to promote the transporter activity, resulting in further increased rate of glucose transport (Konrad et al., 2001). We demonstrated that both PI3K and p38 MAPK activation are required for stimulation of glucose transport by WE in which p38 MAPK-dependent pathway is more important to the transport process compared to the PI3K-dependent pathway because SB 203580 markedly prevented the action of WE, where as the inhibitory effect of wortmannin towards the PI3K activity was small. Slight gain in the rate of glucose uptake in response to a combination treatment around 124% over the rate by insulin alone (Figure 3) supports that the mechanism by which WE stimulates the glucose transport is mainly similar to that employed by insulin. A 5′-AMP-activated kinase (AMPK) activation is also implicated as a mechanism for the induction of skeletal muscle glucose transport (Hayashi, Hirshman, Kurth, Winder, & Goodyear, 1998). This effect is independent to insulin-responsive pool of glucose transporter, and the GLUT4 translocation through AMPK activation is not affected by wortmannin (Li, Randhawa, Patel, Hayashi, & Klip, 2001; Yamaguchi et al., 2005). Additional studies will be required to further elucidate whether the effect of WE to increase insulin action may attribute to AMPK as well.

Figure 4. Effects of the water extract of *Mitragyna speciosa* leaves (WE) on GLUT1 and GLUT4 total protein content in L8 myotubes. Cells were incubated with and without 0.6 mg mL$^{-1}$ WE for 1 or 6 h. Whole cell lysates were separated on SDS-PAGE, electrophotected, and probed with antibodies specific for GLUT1 and GLUT4. Data shown are from one independent experiment.
The total content of GLUT1 and GLUT4 was not changed after short exposure to the WE. This indicates that the steady state of the transporter was not affected. GLUT1 is the main glucose transporter of L8 cells (Wertheimer, Sasson, Cerasi, & Ben-Neriah, 1991). In the presence of high glucose, the content of GLUT1 mRNA is reduced, resulting from increased muscle glucose-6-phosphate and decreased glucose disposal (Sasson et al., 1997; Young, Bogardus, Stone, & Mott, 1988). In this present study, we tested the effect of WE in 15 mM glucose, a level corresponding to that observed in the hyperglycemia. We have shown a slight increase in GLUT1 protein level after 6 h exposure despite the expected down-regulatory effect of GLUT1 mRNA under this condition. The result implies that WE may be involved in the post-transcriptional level of GLUT1 gene expression that contributes to increased amount of cell surface transporter and consequently the rate of glucose uptake.

The observation that all extracts of Kratom and mitragynine were active in the evaluating range and a similar extent of response was achieved from ME at dose lower than that of mitragynine leads us to believe that mitragynine together with some other compounds act in concert to modulate the transport of glucose into cells. Besides mitragynine and other alkaloids, phenolic compounds were also detected in ME and WE (screening with 1% FeCl₃ solution), which remain to be identified and assessed for the observed activity.

In summary, M. speciosa or Kratom exhibits an insulin-like action on stimulation of glucose transport. Acute M. speciosa treatment stimulates glucose uptake in L8 muscle cells in part by activating GLUT4 redistribution to plasma membrane via PI3K-dependent pathway without changing amount of GLUT4. It is possible that M. speciosa may upregulate GLUT1 protein, hence increased GLUT1 protein content plays an important role in activating the transport in long-term exposure. Mitragynine is one of several activators in M. speciosa that activates glucose transport in muscle cells. These results lend some support to the folkloric use of M. speciosa leaves for treating diabetes. The anti-diabetic effect of M. speciosa extract is being investigated in the animal model.

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References


