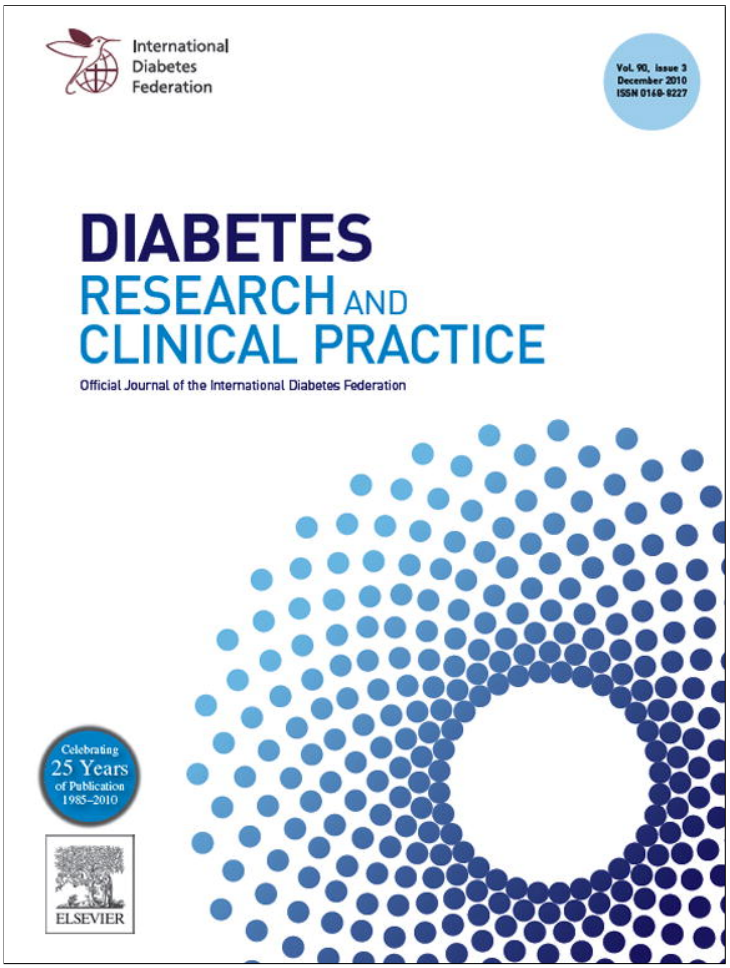


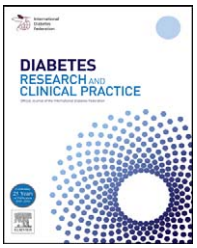
DIABETES RESEARCH AND CLINICAL PRACTICE 90 (2010) e67–e71

Contents lists available at ScienceDirect

Diabetes Research and Clinical Practice

journal homepage: www.elsevier.com/locate/diabres

International Diabetes Federation



Brief report

Carbazoles and coumarins from *Clausena harmandiana* stimulate glucose uptake in L6 myotubes

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ARTICLE INFO

Article history:
Received 15 June 2010
Received in revised form
26 August 2010
Accepted 2 September 2010

Keywords:
Clausena harmandiana
Myotubes
Carbazole
Coumarin
Diabetes

ABSTRACT

Two carbazoles (compounds **1** and **2**) and one coumarin (compound **8**) from *Clausena harmandiana* exhibited significant glucose uptake activity in L6 myotubes in a time and dose dependent manner. In addition, compounds **2** and **8** were inhibited by p38 mitogen-activated protein kinases and phosphatidylinositol 3-kinases, respectively.

1. Introduction

Clausena harmandiana is an endemic species that grows in deciduous forests in Thailand. The young leaves are used as a vegetable in traditional dishes in northeastern Thailand and as fodder for cattle and buffalo. It is called ''Song Fa'' in Thai and has shown some therapeutic activities in stomach ache, headache, sickness and as a health promoting herb. In previous investigations of this plant, carbazoles and coumarins have been isolated and evaluated for many activities [1,2]. There are many reports about the biological activities of carbazoles and coumarins from many plants. The biological activities of carbazoles have been evaluated as antitumor, antioxidative, antimutagenic, anti-inflammatory, anti HIV activities and that they induce apoptosis in a leukemia cell line [3,4]. It has been reported that coumarins exhibit antibacterial, antitumor promoting actions and have an inhibitory effect on iNOS protein expression [5]. However, there is no report on the effect of this plant in the field of diabetes.

In healthy humans, blood-sugar level is kept at a concentration of around 5 mM. This mechanism is maintained

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0168-8227/$ – see front matter
doi:10.1016/j.diabres.2010.09.005

by a well-known hormone, insulin, which stimulates blood glucose uptake into the peripheral tissue, muscles and adipocytes [6]. In skeletal muscle, glucose transport can be activated *via* at least two separate mechanisms, both insulin-dependent and insulin-independent. It is now clear that the stimulation of glucose uptake by insulin is mediated by translocation [7,8] and intrinsic activation of glucose transporter 4 (GLUT4) [9]. Phosphatidylinositol 3-kinases (PI3-kinase) and p38 mitogen-activated protein kinases (p38 MAPK) are the key enzymes regulating GLUT4 translocation and intrinsic activation. In addition to regulation of the GLUT4, glucose uptake is also modulated through changes in the transporter's biosynthesis and protein stability [10,11].

The present study aimed to explore the effect of crude extracts and all isolates from *C. harmandiana* on glucose transport in L6 myotubes, as well as elucidation of its cellular mechanisms through the insulin-dependent signaling pathway.

2. Methods

2.1. Extraction and purification

Air-dried and finely powdered roots (1.3 kg) of *C. harmandiana* were sequentially extracted at room temperature for 3 days with hexane, ethyl acetate and methanol. The crude hexane extract was subjected to column chromatography on silica gel 60 and subsequently eluted with a gradient of three solvents (hexane, EtOAc and MeOH) resulting in 13 groups of eluting fractions which were designated as F1–F13. Purification of F7 was carried out on silica gel column and eluting with the three gradient systems above to give **1** (2.86 g). Further rechromatography of F8 afforded **2** (4.29 g) and **3** (3.25 g) while F9 furnished compounds **4** (1.17 g) and **5** (1.56 g). Compounds **6** (55 mg) and **7** (19 mg) were obtained from the purification of F11. The crude ethyl acetate extract was further purified by column chromatography, affording **8** (90 mg), **9** (17 mg), **10** (12 mg), **11** (4.8 g) and **12** (2.2 g).

2.2. Cell culture and 2-deoxyglucose uptake assay

L6 myotubes were purchased from ACTT (ATCC, CRL-1458). The production of L6 cell cultures and the induction of differentiation were performed as described by Klip et al. [12]. L6 myotubes were maintained in α-MEM containing 10% Fetal Bovine Serum (FBS) and 1% antibiotic solution (penicillin–streptomycin) in an atmosphere of 5% CO_2 at 37 °C and rendered quiescent in α-MEM containing 2% Horse Serum (HS) for 7–8 days to promote fusion into myotubes. 2-Deoxyglucose uptake was measured as described earlier [13]. All compound stock solutions were prepared using DMSO and subsequently diluted with α-minimum essential medium (MEM) before application to the cells. Metformin was dissolved in α-MEM. Cell viability was evaluated by spectrophotometric analysis using MTT [3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyl-tetrazolium bromide] [14].

2.3. Mechanism study using inhibitors

The insulin-dependent signaling pathway associated with uptake was chosen for this study. In this series of experiments, phosphatidylinositol 3-kinases inhibitor (wortmannin; WN), p38 mitogen-activated protein kinases inhibitor (SB203580) and protein synthesis inhibitor (cycloheximide; CHX), were employed either alone or in combination with either 2 mM metformin or either compounds **1** (50 μM), **2** (50 μM), or **8** (25 μM), which significantly stimulated glucose uptake in L6 myotubes. For the inhibitor studies condition, 3.5 μM CHX [15], 1 μM WN [16], 10 μM SB203580 [17] were added individually or in combination to the metformin or compounds-treated cells at fixed times (CHX, 24 h; WN, 30 min; and SB203580, 30 min) prior to the end of the 24-h incubation.

2.4. Statistical analysis

All results were expressed as mean ± SE. Statistical comparisons were tested using student's *t*-test and the differences were accepted as significant at the level of $p < 0.05$.

3. Results

The crude hexane, EtOAc and MeOH extracts were tested for glucose uptake activity and it was found that the crude hexane extract was the most potent part followed by EtOAc and MeOH extracts. Activity guide fractionation of the crude hexane extract led to the isolation of five carbazoles; heptaphylline (**1**), 7-methoxyheptaphylline (**2**), 7-methoxymukonal (**5**), 7-hydroxyheptaphylline (**6**) and clausine C (**7**), as well as two coumarins; dentatin (**3**) and xanthoxyletin (**4**). In the same manner, the purification of the crude EtOAc extract afforded a coumarin; nordentatin (**8**), and 4 carbazoles; 7-methoxymurrayanine (**9**), clausine E (**10**), lansine (**11**) and clausine K (**12**). The structures of all compounds were identified by spectroscopic methods (IR, 1D and 2D NMR, HRMS) together with the comparison of their spectroscopic data with literature values. The chemical structures of all isolated compounds are shown in Fig. 1.

Among 12 isolates, it was found that three of them, **1**, **2** and **8** showed significant increases in glucose uptake in the L6 myotubes ($p < 0.05$ and $p < 0.01$). These three compounds were thus selected for further study on dose and time dependent activity and the results are shown in Fig. 2A and B. The mechanism of glucose uptake was studied by using cytoskeleton inhibitor (cytochalasin B), phosphatidylinositol 3-kinase (wortmanin), p38 MAPK inhibitor (SB203580) as well as protein synthesis inhibitor (cycloheximide) and the results are shown in Table 1.

It was found that compounds **1**, **2** and **8** showed significant increases in glucose uptake with 50 μM of **1** and **2** (195.48 ± 18.86% and 212.72 ± 22.98% above basal; $p < 0.05$, respectively) while **8** showed the most significant increases in glucose uptake at 25 μM (and 425.62 ± 56.50%; $p < 0.001$) (Fig. 2A). Appropriate concentrations of **1** (50 μM) and **2** (50 μM) increased glucose uptake in L6 cells to significant levels within 8–24 h of incubation while the stimulation of glucose uptake by 25 μM of **8** was initially observed after 30 min incubation (Fig. 2B). Cell viability tests confirmed that all three compounds, at concentrations sufficient for enhancement of glucose transport, did not affect cell viability (Fig. 3). The effect of prolonged (24 h) treatment with these three compounds on

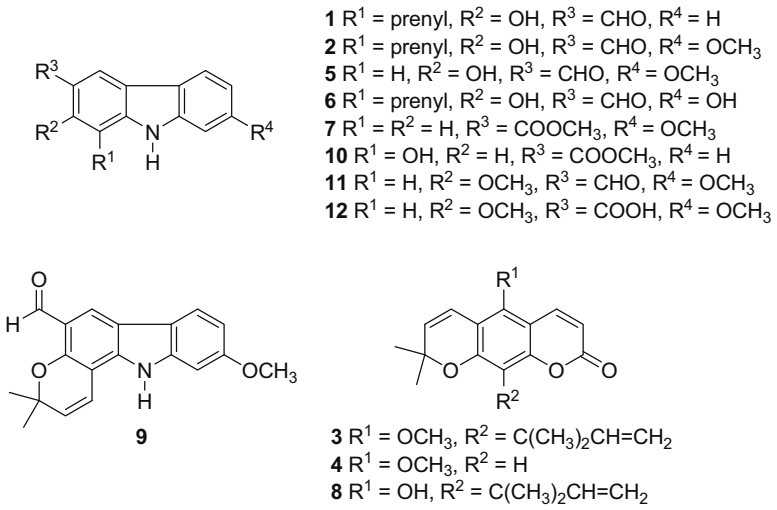

Fig. 1 – Chemical structures of all isolated compounds (1–12).

glucose transport in L6 myotubes was compared with the known antiglycemic drug, metformin. As shown in Table 1, **1** (50 μM), **2** (50 μM), **8** (25 μM) and metformin (2 mM) significantly increased the 2-DG uptake (192.14 ± 6.34%, 205.44 ± 3.42%, 468.48 ± 36.21% and 265.26 ± 1.74 above basal, respectively, $p < 0.01$). The enhancement of glucose transport activity was completely abolished by a cytoskeleton inhibitor, 10 μM of cytochalasin B (remaining 8.41 ± 1.74 compared to basal), suggesting an active process of glucose transport (Table 1). We next undertook a preliminary exploration of the mechanisms involved in the glucose transport activity of these three compounds. The specific inhibitor of the PI3-kinase (wortmannin) was used to determine the involvement of these three compounds on the translocation activity of GLUT4 in L6 myotubes. The intrinsic activity of GLUT4 is activated by p38MAPK activation and this activity was inhibited by

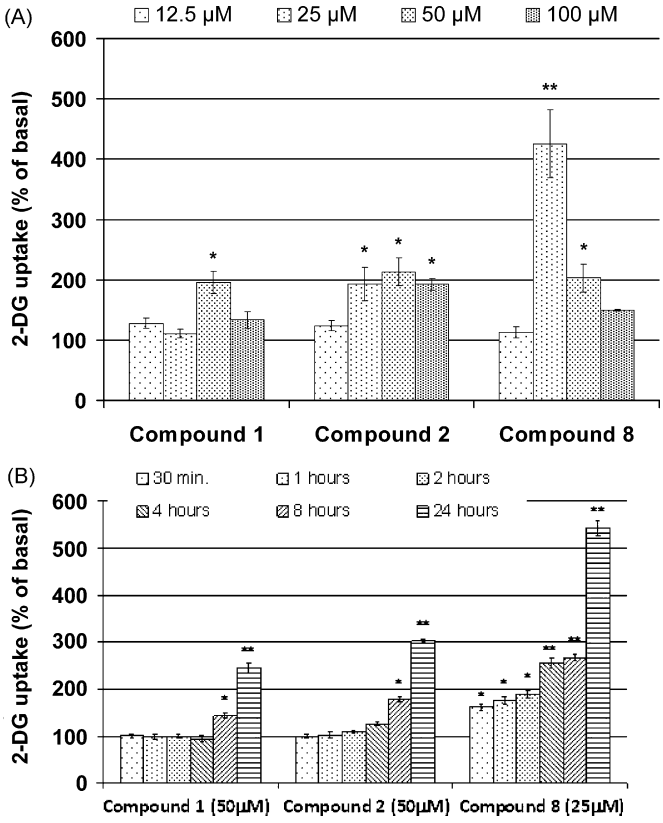

Fig. 2 – Dose and time dependent stimulation of glucose uptake by 1, 2 and 8 in L6 myotubes. Cells were co-incubated with (A) various concentrations of three compounds for 24 h and (B) 50 μM of 1, 50 μM of 2 and 25 μM of 8 at various time-intervals.

Table 1 – The effect of specific inhibitors on compound-stimulated 2-DG uptake.

Inhibitor	2-Deoxyglucose uptake (% of basal)				% Stimulation by				
	Basal control	**1**, 50 μM	**2**, 50 μM	**8**, 25 μM	2 mM metformin	**1**	**2**	**8**	Metformin
None	100.00	192.14 ± 6.34	205.44 ± 3.42	468.48 ± 36.21	265.26 ± 1.74	92.14	105.44	366.48	165.26
10 μM Cyto B	8.41 ± 1.74	7.86 ± 0.83	6.95 ± 1.56	13.57 ± 1.72	8.24 ± 1.51	0	0	0	0
10 μM SB203580	94.62 ± 5.27	192.30 ± 13.61	164.58 ± 11.69	442.71 ± 47.27	255.39 ± 11.41	97.68	69.96[a]	348.09	160.77
1 μM WN	72.29 ± 3.11	183.70 ± 15.98	169.49 ± 10.73	358.89 ± 18.03	254.56 ± 3.00	111.41	97.20	286.60[a]	182.27
3.5 μM CHX	45.29 ± 4.48	102.66 ± 8.84	119.14 ± 12.63	191.87 ± 20.16	171.25 ± 8.37	57.37[a]	73.85[a]	146.58[a]	125.96[a]

Results represent the mean ± SE of three independent experiments carried out in triplicate.
Cyto B = cytochalasin B; WN = wortmannin; CHX = cycloheximide.
[a] $p < 0.05$ compared to the inhibitor-untreated cells.

SB203580 inhibitor. In Table 1, 10 μM SB203580 significantly inhibited compound **2** for stimulated glucose uptake by 35.48% (declining from 105.44% to 69.96%) but had no effect on **1** and **8**. As shown in Table 1, the stimulation of glucose transport by 8 alone was significantly decreased by wortmannin (declining from 366.48% to 286.60%). To determine whether these three compounds induced 2-DG uptake accompanied by an increase in the amount of new protein synthesis, 24-h pre-incubated cells either treated or untreated with protein synthesis inhibitor (cycloheximide, CHX) were subjected to the 2-DG uptake assay. In the presence of 3.5 μM CHX, the 2-DG uptake trails mediated by **1**, **2** and **8** declined from 192.14 ± 6.34% to 102.66 ± 8.84%, 205.44 ± 3.42% to 119.14 ± 12.63% and 468.48 ± 36.21% to 191.87 ± 20.169%, respectively (Table 1).

4. Discussion

In the present study, our results show that both carbazoles and coumarins from *C. harmandiana* stimulate basal glucose uptake in L6 myotubes, much more prominently than metformin. It has been reported that methanolic extract of *C. lansium* is able to decrease blood glucose level in glucose loaded hyperglycemic mice and increase insulin release from INS-1 cells [18]. Coumarin, a naturally phenolic substance presented in a wide variety of plants, has also been reported to reduce the blood glucose levels in diabetic rats [19–21]. Carbazole alkaloid isolated from *Murraya koenegii* leaves has been shown to improve hyperglycemia in diabetic golden hamsters [22]. Because glucose uptake in insulin-sensitive tissues, including muscle and fat cells, is a critical step in maintaining glucose homeostasis and in clearing the post-prandial glucose load [6], our results may explain partially the mechanism of *C. harmandiana* on hypoglycemic effects.

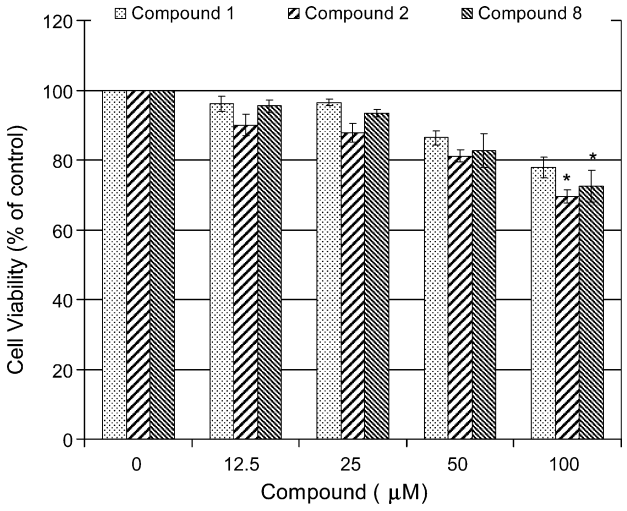

Fig. 3 – Effects of compounds 1, 2 and 8 on the viability of L6 cells. Cell viability was measured by the MTT assay. L6 cells were exposed to various concentrations of compounds (12.5–100 μM) for 24 h. Each point represents the mean with SE of three independent experiments and *$p < 0.05$ compare to control.

To understand the mechanism underlying the stimulation of glucose transport by carbazoles and coumarins isolated from *C. harmandiana*, we examined the effect of these compounds on the insulin-dependent signaling pathway in L6 myotubes. Previous literature has documented that the PI3-kinase pathway plays an important role in the insulin signaling cascade leading to glucose transport translocation [23]. In our study, glucose uptake induced by coumarin (compound **8**) was associated with the activation of PI3-kinase. Pretreatment of L6 myotubes with a specific PI3-kinase inhibitor, wortmannin, decreased coumarin-induced glucose uptake. Therefore, glucose transport and transporter translocation induced by coumarin depends on the involvement of PI3-kinase.

It is now well established that insulin can stimulate glucose uptake into muscle cells employing the p38 MAPK-dependent pathway since this enzyme can be inhibited by SB203580 [24]. However, the mechanism of the action of *C. harmandiana* extracts on glucose uptake into L6 myotubes is still unclear. In the present study, SB203580 has a significant inhibitory effect on carbazole **2** induced glucose uptake but not by carbazole **1**, suggesting that the activation of p38 MAPK is mediated by **2**.

In this present study, the data shows that cycloheximide blocks the stimulation of glucose uptake induced by carbazoles alkaloids, and coumarins. Additionally, extracts from *Aegles marmelos*, *Syzygium cumini* and *Canna indica*, plants rich in flavonoids, stimulate glucose uptake and these effects are totally inhibited in the presence of cycloheximide, suggesting that active protein synthesis is important in terms of glucose transport [25,26]. Thus, these data indicate that active protein

synthesis is necessary for carbazoles and coumarins-stimulated glucose transport.

In conclusion, the results have clearly demonstrated carbazoles and coumarins isolated from *C. harmandiana* increases basal glucose transport in muscle cells by increasing new protein synthesis as well as translocating and regulating the intrinsic activity of GLUT4. Collectively, the plant must be considered as an excellent candidate for future studies on diabetes. In addition, further comprehensive pharmacological investigations, including experimental chronic studies, should be carried out.

Acknowledgements

We thank the National Research Council of Thailand for financial support. The Center for Innovation in Chemistry (PERCH-CIC), Commission on Higher Education, Ministry of Education is gratefully acknowledged. The high resolution mass spectra were provided from Chulabhorn Research Institute, Bangkok, Thailand.

Conflict of interest

The authors declare that they have no conflict of interest.

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