Screening of Plant Constituents for Effect on Glucose Transport Activity in Ehrlich Ascites Tumour Cells

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The effect of plant extracts on D-glucose uptake by Ehrlich ascites tumour cells was examined. Among the 23 exracts of medicinal plants, five samples inhibited, and six samples activated, the uptake significantly. From one of the active plants, *Lagerstroemia speciosa*, two triterpenoids, colosolic acid and maslinic acid were isolated. Colosolic acid was shown to be a glucose transport activator. Since this compound was known to have hypoglycemic activity, our simple *in vitro* bioassay method can at least be used as a first screening for anti-diabetic activity.

Keywords glucose transport; antidiabetic; *Lagerstroemia speciosa*; Lythraceae; triterpene; colosolic acid

Glucose transport is one of the most important functions of all cells to acquire energy. Several types of a glucose transporter are known in cell membranes of mammalian tissues. Glucose transporter is important in regulating the level of intracellular glucose.[1)]

Modification of the activity of glucose transport would cause several physiological effects, *i.e.*, lowering blood glucose level, *etc.* Up to now, only a few compounds have been known to affect glucose transport activity. The compounds which inhibit glucose transporter activity are forskolin,[2)] diterpene isolated from Labiatae plant, phlorezin,[3)] dihydrochalcone of Rosaceae and cytochalasin B,[4)] one of the mycotoxins. On the other hand, no other agent able to increase glucose transport activity is known except insulin, a pancreatic hormone, which regulates blood sugar intrinsically.

Systematic research in the pursuit of an agent to modify glucose transport activity has been carried out in search for a new type of agent for the treatment of diabetes, a tonic for the aged, *etc.* In particular, finding of activator is more important.

In this report, we describe the establishment of a screening method for measuring glucose transport activity which can be used for rapidly evaluating many types of sample, ranging from crude extracts to pure compounds.

Ehrlich ascites tumour cells were used to measure glucose transport activity because these cells are known to contain a glucose transporter,[5)] and they can be easily propagated and used as an experimental system without the need for a complex procedure to separate cells, which might injure the cellular membranes.

The finding of a glucose transport activator in plant extracts and the isolation of an active principle from one of these active plant extracts are also described.

Results and Discussion

The time course of 2-deoxy-D-glucose (2-DG) uptake by Ehrlich cells was measured (Fig. 1). The rate of uptake was linear up to 2 min at concentration of 0.2—1.0 mM. Accordingly, experiments with test solutions were carried out using an incubation time of 1 min. Under these experimental conditions, the K_m and V_{max} values were 1.7 mM and 1.4 nmol/min/10^6 cells, respectively, calculated from Lineweaver–Burk plots. The K_m value obtained was consistent with the reported value for type 1 glucose transporter.

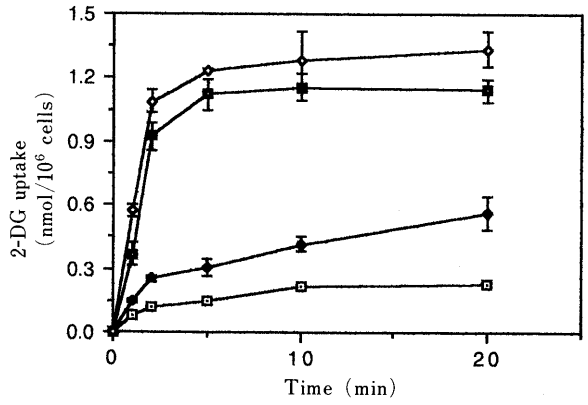


Fig. 1. Time-Course of 2-DG Uptake by Ehrlich Cells

The uptake was measured at 37 °C at final concentrations of 2-DG; □, 0.1 mM; ◆, 0.2 mM; ■, 0.5 mM; ◇, 1.0 mM. Each point represents the mean ± S.E. of three experiments performed in triplicate.

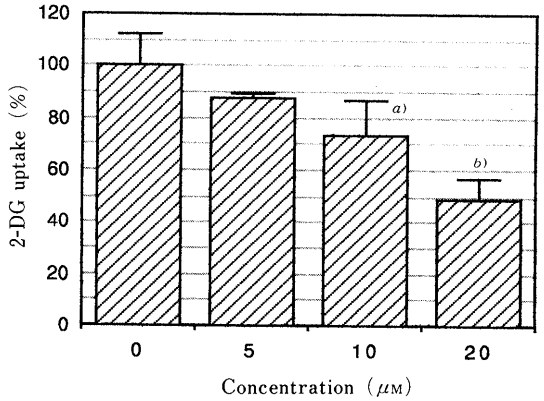


Fig. 2. Effects of Forskolin on 2-DG Uptake (% of Control) by Ehrlich Cells

The uptake was measured at 37 °C. Each bar represents the mean ± S.E. of three experiments performed in duplicate. *a*) $p<0.02$, *b*) $p<0.01$.

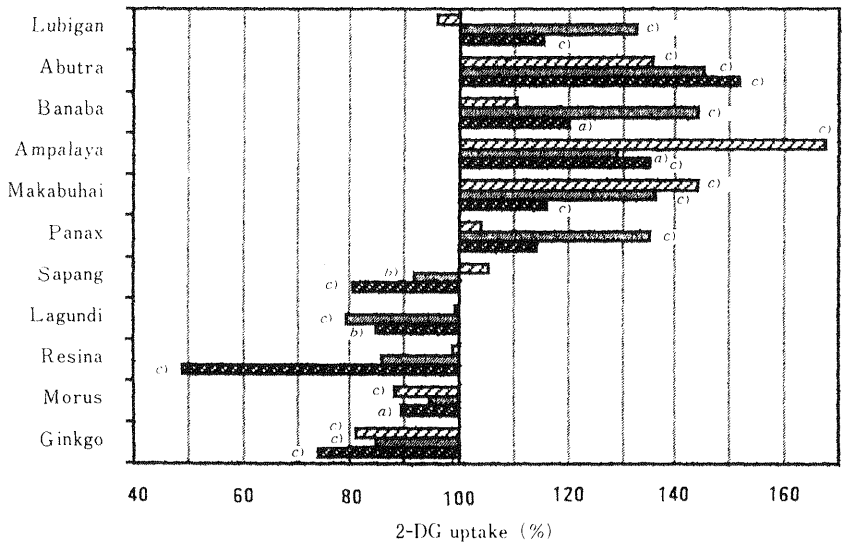


Fig. 3. Effects of MeOH Extracts of Medicinal Plants on the Uptake of 2-DG by Ehrlich Cells (Significant Activity Only)

X axis: % of the control value. Each bar represents the mean of three experiments performed in duplicate determinations. ▨, 1 μg/ml; ▨, 5 μg/ml; ▨, 10 μg/ml. *a*) $p<0.05$, *b*) $p<0.02$, *c*) $p<0.01$.

TABLE I. Tested Plants (from the Philippines)

Local name	Scientific name	Family	Part used
Lubigan	*Acorus calamus*	Araceae	Rhizome
Abtra	*Alcangelisia flava*	Menispermaceae	Bark
Sambong	*Blumea balsamifera*	Compositae	Leaves
Sapang	*Caesalpinia sapan*	Leguminosae	Xylem
Kalingag	*Cinnamomum mercadoi*	Lauraceae	Bark
Anonang	*Cordia myxa*	Boraginaceae	Bark
Tsaang-gubat	*Ehretia microphylla*	Boraginaceae	Leaves
Gogo	*Entada phaseoloides*	Leguminosae	Bark
Banaba	*Lagerstroemia spesiosa*	Lythraceae	Leaves
Amparaya	*Momordica charantia*	Cucurbitaceae	Leaves
Bangkal	*Nauclea orientalis*	Rubiaceae	Leaves
Pandan	*Pandanus* sp.	Pandanaceae	Xylem
Makabuhai	*Tinospora rumphii*	Menispermaceae	Stem
Lagundi	*Vitex negundo*	Verbenaceae	Leaves
Resina	Unidentified		Resin

TABLE II. Tested Plants (from Japan)

Local name	Scientific name	Family	Part used
Rensen-so	*Glechoma hederaceae*	Labiatae	Whole
Sou-boku	*Aralia elata*	Araliaceae	Radix
Araragi	*Taxus cuspidata*	Taxaceae	Xylem
Souhaku-hi	*Morus alba*	Moraceae	Cortex
Suika-hi	*Citrullus vulgalis*	Cucurbitaceae	Peel
Nanka-shi	*Cucurbita moschata*	Cucurbitaceae	Seeds
Ginseng[a)]	*Panax ginseng*	Araliaceae	Radix
Ginkgo[a)]	*Ginkgo biloba*	Ginkgoaceae	Leaves

a) Standardized extract obtained from Switzerland.[8)]

The effect on glucose transport activity of forskolin, a known glucose transport inhibitor, was measured in this system: it inhibited 2-DG uptake at a concentration of 20 μM by 51% (Fig. 2). Forskolin can accordingly be used as a control in our system.

Then, the effect of 23 methanolic extracts of medicinal plants on glucose transport activity was measured at three different concentrations. The plants were randomly chosen from Philippine herbal medicine (Table I), and Japanese medicinal plants used mainly for the treatment of diabetes (Table II). Among the 23 samples, 6 samples accelerated 2-DG uptake and 5 samples reduced it, while the others were ineffective. (Fig. 3)

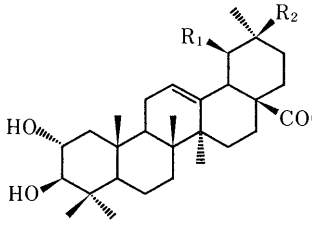
Although both effects were interesting, we focused on stimulation in this report. Since, as mentioned above, no stimulating agent of glucose transport has been reported except insulin. In addition, among the plants exhibiting a positive effect, *Lagerstroemia speciosa* and *Momordica charantia* were used as antidiabetic agents in the Philippines,[6)] and the hypoglycemic effect of *Tinospora cordifolia* (syn. *T. rumphii*) has recently been reported.[6,7)] Preliminary results of the effect of ginseng extract have also been reported.[8)] These screening results prompted us to study the active principles of these glucose transport-stimulating plants.

The target plant, *Lagerstroemia speciosa* L. is distributed all over the Philippines, as well as in India, Malesia, South China and tropical Australia. The leaves of this plant are called "Banaba" in the Philippines, and used as an antidiabetic; the decoction has been clinically tested and found to reduce blood sugar.[9)]

The bioactive MeOH extract of Banaba was fractionated and subjected to column chromatography. The bioactivity of each fraction was monitored at each stage of the isolation processes. From the active MeOH fraction eluted from a Diaion HP-20 chromatography column, compounds **1** and **2** were isolated by silica-gel column chromatography in yields of 0.01 and 0.0016%, respectively. Compounds **1** and **2** were identified by means of NMR as known triterpenes, colosolic acid (2α-hydroxyursolic acid) and maslinic acid (2α-hydroxyoleanolic acid), respectively.[10,11)]

The bioactivity of **1** and **2** was measured by the above method. Colosolic acid (**1**) showed a significant glucose transport-stimulating activity at a concentration of 1 μM, while **2** was inactive (Table III).

The hypoglycemic effect of **1** has recently been reported in normoglycemic rats following oral administration.[12)] This evidence strongly suggests that our *in vitro* bioassay



	R_1	R_2
1	CH_3	H
2	H	CH_3

Chart 1

TABLE III. Effects of Two Triterpenes Isolated from *Lagerstroemia spesiosa* on the Uptake of 2-DG in Ehrlich Cells (%)

Concentration (μM)	Compounds	
	1	**2**
0	100.0± 4.1	100.0±4.1
1	127.9±10.9[a)]	107.1±5.6
10	109.8± 7.7	99.4±3.9
20	107.9± 6.1	99.1±5.1

Measured at 37 °C after 1 min. Each figure represents the mean±S.E. of three experiments performed in triplicate. *a)* $p<0.03$.

is closely related to the hypoglycemic effect and may be used as a first screening method for anti-diabetic substances without the need for many animals, as in an *in vivo* assay. Examination of the correlation of both activities and a further search for active substances in other plants are in progress.

Experimental

Plant Materials Sixteen crude drugs were purchased at Quiapo Market in Manila, Philippines. They were identified by Dr. Cantoria, Professor of Pharmacognosy, Faculty of Pharmaceutical Sciences, University of the Philippines. Six Japanese crude drugs were obtained in Yamamoto Pharmacy, Hiroshima, Japan. Ginseng and Ginkgo extract are gift from Pharmaton Co. Ltd., Switzerland.

Preparation of MeOH Extracts Material from each plant (5 g) was extracted with boiling MeOH (50 ml × 3) and concentrated to dryness. Assay samples were prepared by dissolving each extract in 0.1% EtOH in Krebs Ringer Hepes (KRH) buffer to give concentrations of 1, 5 and 10 μg/ml.

Cell Suspension Ascites fluid (about 5 ml) was taken from a ddY mouse (15—25 g) 10 d after inoculation with Ehrlich ascites tumour cells (4—5 × 10^7 cells). This was suspended in KRH buffer and centrifuged twice at 800 rpm for 5 min twice to remove the ascites fluid. The precipitate obtained was suspended in KRH buffer to give a cell suspension at a concentration of 1—2 × 10^8 cells/ml (counted under a microscope).

Measurement of 2-DG Uptake Transport of D-glucose into cells was measured by a rapid filtration technique as previously described.[8)] An aliquot of 20 μl of the above cell suspension was suspended in 230 μl of KRH buffer with 50 μl of the sample solutions and was incubated at 37 °C for 5 min. Then the reaction was initiated by adding 20 μl of 2.5 mM 2-DG containing [^{3}H]-2-DG (1.5 μCi) and the mixture was further incubated for 1 min. The reaction was terminated by filtering through glass microfiber filters (2.5 cm, Whatman) and rapidly washing 3 times with 3 ml of KRH buffer. The radioactivity remaining on the filter was determined by liquid scintillation spectrometry.

Statistical Analysis Results were given as means±S.E. for the indicated number of independently performed experiments. Differences between the mean values were examined by Student's *t*-test.

Bioassay-Guided Isolation The origin of the plant, *Lagerstroemia speciosa* PERS. was as mentioned in Plant Materials. Air dried leaves (310 g) were extracted with boiling MeOH (2 l × 3). The extract (18 g) which showed +35% glucose transport activity at 5 μg/ml, was suspended in water and extracted with ether to yield 7.7 g of ether soluble material which showed −17% activity. The aqueous fraction was separated on Diaion HP-20 column chromatography eluting with water, MeOH and acetone to give three fractions at yields and activity (at 5 μg/ml) of 5.3 g (+21%), 4.8 g (+35%) and 325 mg (0%), respectively. The most active MeOH fraction as further chromatographed on silica gel followed by HPLC on ODS-120A and ODS-120T to afford **1** (31 mg) and **2** (5 mg).

Colosolic Acid (1) A white powder, $[\alpha]_D^{16}=+36°$ (MeOH, $c=0.18$). ^{1}H-NMR (C_5D_5N, 400 MHz) δ: 5.46 (1H, brs, H-12), 3.40 (1H, d, $J=9.5$ Hz, H-3α), 2.63 (1H, d, $J=11.3$ Hz, H-18β), 1.27, 1.21, 1.08, 1.05, 0.98 (each 3H, s, 5 × CH_3), 0.99 (3H, d, $J=6.2$ Hz, CH_3), 0.96 (3H, d, $J=5.9$ Hz, CH_3). ^{13}C-NMR (C_5D_5N, 100 MHz) δ: (from C-1 to C-30) 48.0, 68.6, 83.8, 39.4, 55.9, 18.8, 33.5, 40.0, 48.0, 38.4, 23.7, 125.5, 139.3, 42.5, 28.6, 24.9, 48.1, 53.5, 39.5, 39.8, 31.0, 37.4, 29.3, 17.5[a)], 17.0[a)], 17.5[a)], 23.9, 179.9, 17.7[a)], 21.4 ([a)] may be interchanged). These values were essentially the same as cited elsewhere.[10,11)]

Maslinic Acid (2) A white powder, $[\alpha]_D^{14}=+26°$ (MeOH, $c=0.37$). ^{1}H-NMR (C_5D_5N, 400 MHz) δ: 5.48 (1H, brs, H-12), 4.10 (1H, ddd, $J=4.4$, 9.4, 12.0 Hz, H-2β), 3.40 (1H, d, $J=9.4$ Hz, H-3α), 3.30 (1H, dd, $J=4.3$, 14.2 Hz, H-18β), 1.28, 1.27, 1.09, 1.01, 0.99, 0.95 (each 3H, s, 7 × CH_3). ^{13}C-NMR (C_5D_5N, 100 MHz) δ: (from C-1 to C-30) 48.2, 68.6, 83.8, 39.9, 55.9, 18.9, 33.2, 39.9, 47.8, 38.6, 23.7, 122.5, 144.9, 42.2, 28.3, 23.8, 46.7, 42.0, 46.5, 31.0, 34.2, 33.2, 29.4, 17.5[a)], 16.9[a)], 17.7[a)], 26.1, 180.2, 33.2, 24.0 ([a)] may be interchanged). These values were essentially the same as cited elsewhere.[10,11)]

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