Biochimica et Biophysica Acta 1848 (2015) 695–701

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Fungicidal effect of isoquercitrin via inducing membrane disturbance

JiEun Yun [a], Heejeong Lee [a], Hae Ju Ko [b], Eun-Rhan Woo [b], Dong Gun Lee [a,*]

[a] School of Life Sciences, BK 21 Plus KNU Creative BioResearch Group, College of Natural Sciences, Kyungpook National University, Daehak-ro 80, Buk-gu, Daegu 702-701, Republic of Korea
[b] College of Pharmacy, Chosun University, 375 Seosuk-dong, Dong-gu, Gwangju 501-759, Republic of Korea

ARTICLE INFO

Article history:
Received 22 August 2014
Received in revised form 13 November 2014
Accepted 17 November 2014
Available online 21 November 2014

Keywords:
Isoquercitrin
Phytochemical
Antifungal activity
Membrane disturbance

ABSTRACT

Isoquercitrin is a flavonoid isolated from *Aster yomena*, which has been used as a traditional medicinal herb. In the present study, we investigated the antifungal activity and the underlying mechanism of isoquercitrin. Isoquercitrin had a potent effect in the susceptibility test against pathogenic fungi and almost no hemolysis. Propidium iodide and potassium release assays were conducted in *Candida albicans*, and these studies confirmed that isoquercitrin induced membrane damage, thereby, increasing permeability. Membrane potential was analyzed using 3,3′-dipropylthiacarbocyanine iodide [$DiSC_3(5)$], and the transition of membrane potential was indicated by an increased fluorescence intensity. To further analyze these results using model membranes, giant unilamellar vesicles and large unilamellar vesicles that encapsulated calcein were prepared and the detection of calcein leakage from liposomes indicated that membrane was disturbed. We further verified membrane disturbance by observing the disordered status of the lipid bilayer with 1,6-diphenyl-1,3,5-hexatriene fluorescence. Moreover, changes in size and granularity of the cell were revealed in flow cytometric analysis. All these results suggested the membrane disturbance and the degree of disturbance was estimated to be within a range of 2.3 nm to 3.3 nm by fluorescein isothiocyanate–dextran analysis. Taken together, isoquercitrin exerts its fungicidal effect by disturbing the membrane of cells.

1. Introduction

The medicinal properties of numerous herbal plants are widely known and have been used for many years to treat a variety of conditions [1]. They can exert physiological actions on the human body, producing therapeutic effects such as anti-hepatotoxic and anti-cancer activity [2]. In addition, many of the anti-microbial compounds that have been isolated from medicinal herbs have low toxicity and elicit minimal side effects [3]. For this reason, there has been increased interest in the medicinal properties of various herbal plants. Specifically, most plants produce organic biomolecules in response to infections or stress caused by microorganisms to protect themselves as chemical barriers [4].

Phytochemicals are small, organic, naturally occurring biomolecules that are secondary metabolites from plants [5]. Phytochemicals may be effective at combating or preventing diseases due to their antioxidant effects. The major phytochemicals are flavonoids, phenolic compounds, and tannins [6]. Flavonoids, which are phenolic structures that contain one carbonyl group, have been shown to inhibit multiple viruses [7,8]. Phenolic compounds are the simplest bioactive phytochemicals consisting of a single substituted phenolic ring. They possess several biological properties, including anti-microbial, anti-viral, and antioxidant activity. Tannins are polymeric phenolic substances that have anti-infective actions [8].

Aster yomena is a perennial herb that grows mainly in South Korea. It is used as a traditional medicine in the treatment of inflammation, colds, and asthma [9]. Isoquercitrin, a dietary flavonoid, is found in medicinal and dietary plants, including herbs, flowers, and vegetables [10]. It has been shown that isoquercitrin has a wide range of therapeutic properties, including anti-inflammatory, antioxidant, and anti-allergic activities [11]. Recently, other effects of isoquercitrin have been reported. Isoquercitrin can potentially be used to prevent lipid metabolism disorder through activation of the adenosine monophosphate-activated protein kinase [10]. Furthermore, anti-asthmatic activity and the possible use as a diuretic have been demonstrated [12,13]. However, to the best of our knowledge, there has yet been no study demonstrating the effect of isoquercitrin against fungi. Here, we report the antifungal activity of isoquercitrin isolated from *A. yomena* against various fungal strains and demonstrate its mode of action.

Abbreviations: CH_2Cl_2, dichloromethane; EtOAc, ethyl acetate; MIC, minimum inhibitory concentration; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide; $DiSC_3(5)$, 3,3′-dipropylthiacarbocyanine; SD, standard deviation; GUV, giant unilamellar vesicle; ITO, indium tin oxide; LUV, large unilamellar vesicle; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; DPH, 1,6-diphenyl-1,3,5-hexatriene; FITC, fluorescein isothiocyanate; FD, FITC-labeled dextran

* Corresponding author. Tel.: +82 53 950 5373; fax: +82 53 955 5522.
E-mail address: dglee222@knu.ac.kr (D.G. Lee).

http://dx.doi.org/10.1016/j.bbamem.2014.11.019
0005-2736/

2. Materials and methods

2.1. Isolation of isoquercitrin

The aerial parts of *A. yomena* Makino (Asteraceae) were collected and air-dried. Next, 1.9 kg of these aerial parts was extracted three times with methanol (MeOH) under reflux, and 120.1 g of residue was produced. The MeOH extract was suspended in water, which was then partitioned sequentially with equal volumes of dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), and n-butanol (BuOH). Each fraction was evaporated in vacuo to yield CH_2Cl_2 (23.6 g), EtOAc (15.2 g), and n-BuOH (48.8 g) residues and water (48.2 g) extract. Column chromatography (CC) was used, and the EtOAc fraction (10.0 g) was chromatographed over a silica gel column using a gradient solvent system of $CHCl_3$:MeOH (5:1 → 1:3) to give five subfractions (E1–E5). The E2 (3.7 g) subfraction was subjected to MCI® Gel CC eluted with a gradient solvent system of MeOH:H_2O (1:1 → 1:0) to yield six subfractions (E21–E26). The E21 (640.1 mg) subfraction was subjected to RP-18 CC eluted with a gradient solvent system of MeOH:H_2O (1:3 → 1:0) to yield nine subfractions (E211–E219). The E218 (60.3 mg) subfraction was purified by silica gel CC ($CHCl_3$:MeOH = 5:1) to produce isoquercitrin (20.2 mg). The physicochemical and spectral data of isoquercitrin, including ^{1}H NMR, ^{13}C NMR, and HSQC, were identical to those reported in the literature (Fig. 1) [14,15].

Yellow powder; $[\alpha]_D^{20}$ − 85° (MeOH, *c* 0.06); EI-MS *m/z*: 464 [M^+]; ^{1}H NMR (300 MHz, CD_3OD) δ: 7.71 (1H, d, *J* = 2.0 Hz, H-2′), 7.57 (1H, dd, *J* = 8.4, 2.0 Hz, H-6′), 6.86 (1H, d, *J* = 8.4 Hz, H-5′), 6.37 (1H, d, *J* = 1.8 Hz, H-8), 6.19 (1H, d, *J* = 1.8 Hz, H-6), 5.24 (1H, d, *J* = 8.0 Hz, H-1″), 3.70–3.29 (6H, m, H-2″–6″). ^{13}C NMR (75 MHz, CD_3OD) δ: 179.6 (s, C-4), 166.1 (s, C-7), 163.1 (s, C-5), 159.1 (s, C-2), 158.6 (s, C-9), 150.0 (s, C-4′), 146.0 (d, C-3′), 135.8 (s, C-3), 123.3 (d, C-6′), 123.2 (s, C-1′), 117.7 (d, C-2′), 116.1 (d, C-5′), 105.8 (d, C-1″), 104.5 (s, C-10), 100.0 (d, C-6), 94.9 (d, C-8), 78.5 (d, C-3″), 78.2 (d, C-5″), 75.9 (d, C-2″), 71.3 (d, C-4″), 62.7 (t, C-6″).

2.2. Antifungal susceptibility test and hemolytic activity assay

Candida albicans (ATCC 90028) and *Candida parapsilosis* (ATCC 22019) were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). *Malassezia furfur* (KCTC 7744), *Trichophyton rubrum* (KCTC 6345), and *Trichosporon beigelii* (KCTC 7707) were obtained from the Korean Collection for Type Cultures (KCTC). The *M. furfur* was cultured at 32 °C in a modified YM broth (Difco) containing 1% olive oil, and the fungal strains were cultured in YPD broth (Difco) with aeration at 28 °C. Fungal cells (2×10^6/mL) were inoculated into YPD or YM broth with 1% olive oil and then dispensed into microtiter plates at a volume of 0.1 mL/well. The minimum inhibitory concentration (MIC) was determined using a standard microdilution method and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay [16,17]. Following overnight incubation, the growth was measured using a microtiter ELISA Reader (Molecular Devices Emax, CA) by monitoring the absorption at 580 nm. The MIC values were determined by three independent assays.

The hemolytic activity of the compounds was assessed by determining the release of hemoglobin from an 8% suspension of human erythrocytes at 414 nm with an ELISA reader. The percentage of hemolysis was calculated using the following equation: hemolysis (%) = 100 [(Abs_{414nm} in the compound solution − Abs_{414nm}) / (Abs_{414nm} in 0.1% Triton X-100 − Abs_{414nm})] [18].

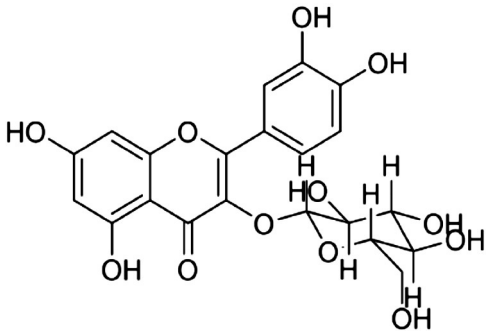

Fig. 1. Structure of isoquercitrin.

2.3. Propidium iodide influx

C. albicans cells (2×10^6/mL), centrifuged at 8000 rpm for 5 min, were suspended in phosphate buffered saline (PBS) and treated with the compounds at a concentration of 2.5 μg/mL. After incubation for 4 h at 28 °C, cells were harvested by centrifugation and suspended in PBS. The cells were then treated with 9 μM propidium iodide and incubated for 5 min at room temperature. The cells were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) [19].

2.4. Potassium release

Potassium release was assessed to examine the change in ion concentration resulting from treatment with the compounds. *C. albicans* cells (OD = 1.0) were resuspended in PBS and treated with the compounds. After incubation for 5-minute intervals at 28 °C, the cells were centrifuged at 13,000 rpm for 10 min. The supernatant was measured using an ion-selective electrode (ISE) meter (Orion Star A214, Thermo Scientific, Singapore). The cells were sonicated to determine 100% potassium release. The percentage of potassium release caused by the compounds was calculated as follows: potassium release (%) = 100 × ($[K^+] - [K^+]_0$) / ($[K^+]_t - [K^+]_0$), where $[K^+]$ represents the potassium release achieved after addition of the compounds and $[K^+]_0$ and $[K^+]_t$ represent the potassium release without the compounds and with sonication, respectively [20].

2.5. Change in membrane electrical potential

To detect the change in the membrane electrical potential, a membrane potential sensitive probe, 3,3′-dipropylthiacarbocyanine iodide [$DiSC_3(5)$] was used. *C. albicans* cells (2×10^6/mL) were centrifuged at 10,000 rpm for 5 min and washed with Ca^{2+} and Mg^{2+}-free PBS. Changes in the fluorescence, caused by the collapse of the membrane potential by 2.5 μg/mL of the compounds, were continuously monitored using a spectrofluorometer (Shimadzu RF-5301PC; Shimadzu, Japan) at an excitation wavelength of 622 nm and an emission wavelength of 670 nm. To ensure reproducibility, the measurement was repeated two times under each condition [21].

2.6. Preparation of giant unilamellar vesicles

Giant unilamellar vesicles (GUVs) were prepared using indium tin oxide (ITO)-coated glass. Lipids, composed of phosphatidylcholine (PC)/phosphatidylethanolamine (PE)/phosphatidylinositol (PI)/ergosterol (5:4:1:2, w/w/w/w), were prepared at a concentration of 3.75 mg/mL in chloroform. The lipid solutions were coated onto an ITO-coated glass for 5 min in a spin coater (Spin Coater, ACE-1020 Series) and then evaporated under vacuum for 2 h. The lipid-coated and uncoated glass were separated by a distance of 2 mm with a thin Teflon spacer. The chamber was filled with 10 mM HEPES buffer (pH 7.2) through a hole in the silicon spacer. The application of a 1.7-V (peak-to-peak) and 10-Hz sine wave was immediately applied to the ITO electrodes for 2 h at room temperature. GUVs from the ITO glass were then detached with the following conditions: 4-V (peak-to-peak) and 10-Hz sine wave for 10 min. The GUVs were treated with the compounds and changes in the GUVs were observed with an inverted microscope (Nikon Eclipse Ti-S, Japan) [22].