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Screening of new chemopreventive compounds from *Digitalis purpurea*

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Received April 27, 2005, accepted June 7, 2005

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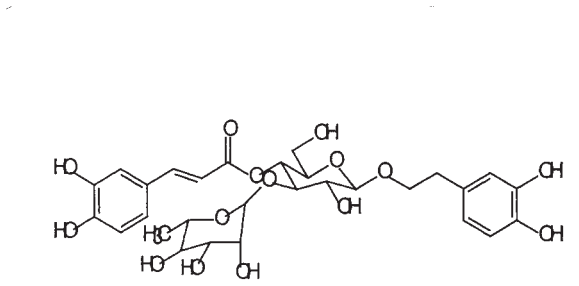
Pharmazie 61: 356–358 (2006)

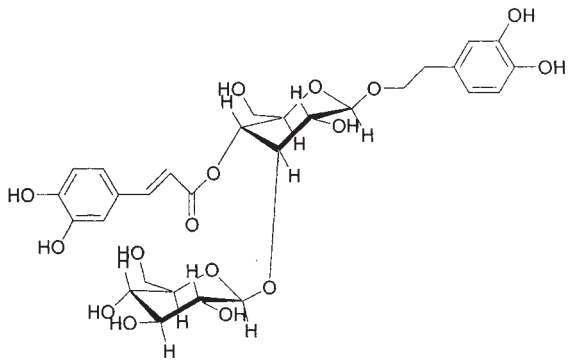
Chemopreventive agents induce a battery of genes whose protein products can protect cells from chemical-induced carcinogenesis. In this study, we isolated four different glycosides (1 acteoside; 2 purpureaside A; 3 calceolarioside B; and 4, plantainoside D) from the leaves of *Digitalis purpurea* and studied their abilities to induce glutathione S-transferase (GST) and their protective efficiencies against aflatoxin B_1-induced cytotoxicity in H4IIE cells. Of these four glycosides, acteoside significantly inhibited the cytotoxicity induced by aflatoxin B_1 (AFB_1) and also selectively increased GSTα protein levels. Reporter gene analysis using an antioxidant response element (ARE) containing construct and subcellular fractionation assays, revealed that GSTα induction by acteoside might be associated with Nrf2/ARE activation. The results suggest that acteoside possesses a potent hepatoprotective effect against AFB_1 and that it can be applied as a potential chemopreventive agent.

1. Introduction

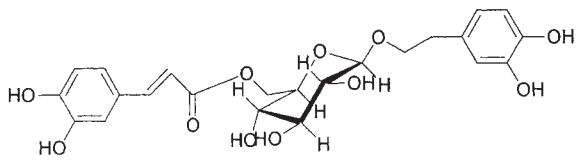
Cancer chemoprevention is defined as the use of naturally occurring compounds to prevent, or reverse the process of carcinogenesis. Moreover, a large body of experimental data indicates the feasibility of this approach. A large number of phytochemicals can act as chemopreventive agents. These include diallyl sulfide from garlic and dithiolthiones from cruciferous plants, which protect tissues and prevent carcinogenesis (Wargovich et al. 1988;



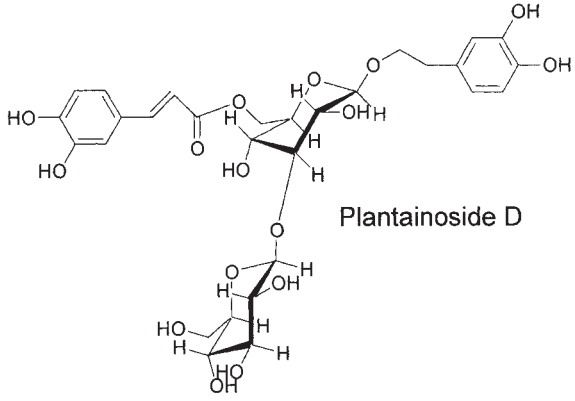
Acteoside



Purpureaside A



Calceolarioside B



Plantainoside D

Wilkinson et al. 1997). These sulfur-containing compounds have been extensively studied as potential chemopreventive agents (Wargovich et al. 1988; Kensler et al. 2004). The one known mode of chemoprevention by phytochemicals involves the induction of phase II detoxifying enzymes such as glutathione S-transferase (GST) (Kensler et al. 1986), and treatment with many chemopreventive agents increases hepatic GST activity by acting through the induction of the gene (Primiano et al. 1995; Guyonnet et al. 1999).

Digitalis purpurea is a perennial herb of the Scrophulariaceae family, and is commonly used to treat congestive heart failure (Willius and Keys 1941). Recently, it was also reported that *Digitalis* glycosides have potent anticancer effects (Lopez-Lazaro et al. 2003). As a part of our program to screen for potential cancer chemopreventive compounds from medicinal plants, we isolated four different glycosides (1 acteoside; 2 purpureaside A; 3 calceolarioside B; and 4, plantainoside D) from the leaves of *Digitalis purpurea* and studied their abilities to induce GST and their protective efficacies against aflatoxin B_1 (AFB_1)-induced cytotoxicity in a rat hepatocyte-derived cell line, H4IIE cells. Furthermore, we also monitored Nrf2/ARE activation in order to investigate the mechanistic basis underlying the induction of GST by the glycosides.

2. Investigations, results and discussion

We isolated four different glycosides from the leaves of *Digitalis purpurea.* First, the effect of each glycoside against AFB_1-induced cytotoxicity was examined in H4IIE cells. Pretreating the cells with acteoside (100 μM, 12 h) significantly inhibited the cell death induced by AFB_1, whereas the other glycosides did not protect cells from AFB_1 (Fig. 1). Earlier studies suggested that the AFB_1-induced formation of ROS or oxidant metabolites plays a critical role in its cytotoxic effect (Shen et al. 1995). This result suggests that the protective effect of acteoside could be due to a reduction in the formation of ROS. It has also been reported that acteoside protects CCl_4-induced hepatotoxicity possibly through the blocking of P450 or the scavenging of free radicals (Lee et al. 2004).

Glutathione (GSH), a nonprotein sulfhydryl intracellular molecule, plays a role as a protective substance and serves as an effective oxygen radical scavenger. GSH-conjugation

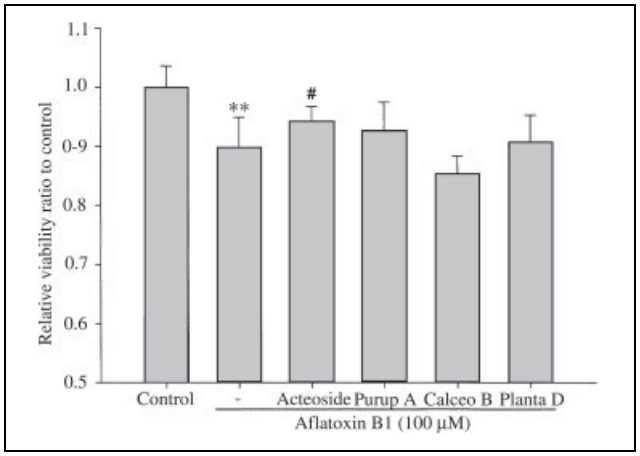


Fig. 1: Effects of the four glycosides on AFB_1-induced cytotoxicity. Cells were serum-starved and incubated in the presence or absence of each glycoside (100 μM) for 12 h, and then treated with various concentrations of AFB_1 for 12 h. Cells viabilities were assessed by MTT assay. Data represent the means ± S.D. of 8 different samples

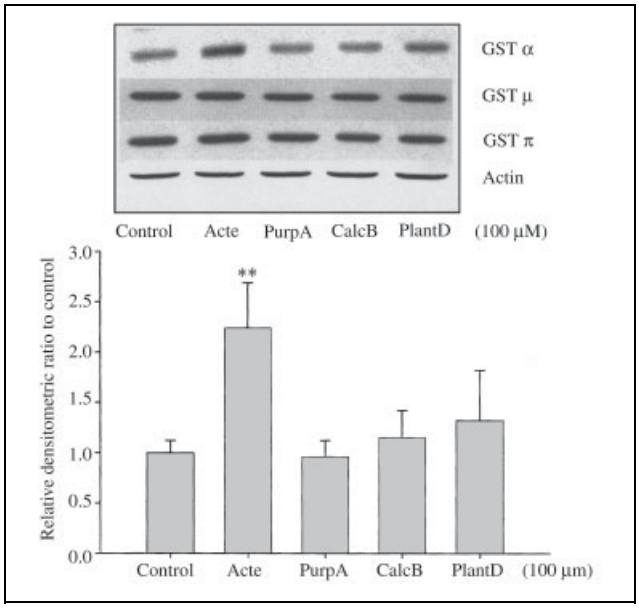


Fig. 2. Effects of the four glycosides isolated from the leaves of *Digitalis purpurea* on GST protein levels. GST subunit protein was monitored 18h after treating cells with each compound (100 μM). Each lane was loaded with 10 μg of cytosolic protein. Data represent the means ± S.D. of 3 separate experiments (significant as compared to control, $^{**}p < 0.01$; control level = 1)

with xenobiotics can be accelerated by GST, and increased GST activity can increase this conjugative reaction and thus lead to AFB_1 detoxification. Hence, the induction of GST is believed to be an important determinant of the characteristics of chemopreventive agents. In the present study, we determined the levels of GST subtypes in H4IIE cells treated with the four digitalis glycosides. Treatment of H4IIE cells with acteoside (100 μM) for 18 h increased GSTα levels, but the other glycosides caused no such increase (Fig. 2), which suggests that the selective induction of GSTα by acteoside seems to be related with its cytoprotective effect against AFB_1. In addition, either GSTμ or GSTπ was not induced by the glycosides (Fig. 2).

The role of antioxidant response elements (AREs) in the inducible expression of phase II enzymes (e.g. GSTα) by several antioxidants and chemopreventive agents has been extensively studied (Kang et al. 2001, 2003). Nrf2 is a key transcription factor that binds to ARE sequences, and which is implicated in the regulation of GST expression (Kang et al. 2001, 2002). To determine whether the induction of GSTα by acteoside is mediated by the activation of Nrf2/ARE, a reporter gene assay was performed using H4IIE cells transfected with the mammalian expression vector pGL-797, which contained the luciferase structural gene and the ARE sequence of GSTA2 promoter (Kang et al. 2003). Exposure of cells transiently transfected with this plasmid, to acteoside resulted in a 6-fold increase in luciferase activity. However, luciferase activity was not noticeably induced by the other 3 glycosides (Fig. 3).

Next, we examined whether acteoside stimulates the translocation of Nrf2 to the nucleus. Subcellular fractionation and Western blot analyses confirmed that acteoside increased the level of Nrf2 in the nuclear fraction at 6–12 h (Fig. 4). These data suggest that the induction of GSTα by acteoside is associated with the activation of Nrf2-mediated ARE activation.

In summary, the present study demonstrates the protective effect of acteoside on AFB_1-induced cytotoxicity. The in-

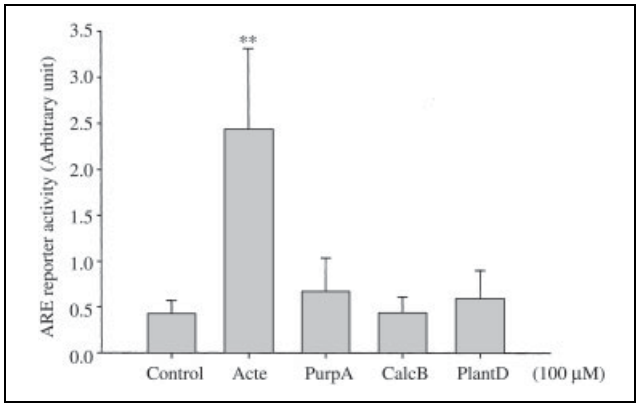


Fig. 3: Induction of luciferase activity by glycosides in H4IIE cells transiently transfected with the GSTA2 chimeric gene construct pGL-797 containing the ARE element. Dual luciferase reporter assays were performed on lysed H4IIE cells co-transfected with pGL-797 (firefly luciferase) and pRL-SV (*Renilla* luciferase) (in a ratio of 100 : 1) after exposure to each glycoside (100 μM) for 18 h. Activation of the reporter gene was calculated as a relative change to *Renilla* luciferase activity. Data represent the means ± S.D. of 4 separate experiments (significant as compared to control, $^{**}p < 0.01$; control level = 1)

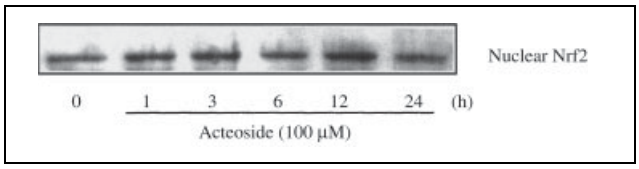


Fig. 4: Nuclear translocation of Nrf2 by acteoside. The subcellular localization of Nrf2 was immunochemically assessed in cells treated with acteoside (100 μM) for 1–24 h. All lanes contained 20 μg of nuclear extracts. Equal loading of protein in each sample was verified using Ponceau-S staining

duction of GSTα by acteoside could contribute to its inhibitory effect on AFB_1-induced hepatocyte damage, presumably by enhancing the GSH conjugation reaction or by enhancing ROS scavenging. Our results suggest that acteoside possesses hepatoprotective effect against AFB_1, and raise the issue of its usefulness as a chemopreventive agent.

3. Experimental

H4IIE cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. Cell viability was measured by MTT assay. Cytosolic and nuclear fraction isolation, immunoblot analysis, and reporter gene assays were performed as previously described (Kang et al. 2003). The paired Student's t-test was used to assess significant differences between the different treatment groups. The criterion for statistical significance was set at either $p < 0.05$ or $p < 0.01$.

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