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Polyphenols from plants used in traditional Indonesian medicine (Jamu): uptake and antioxidative effects in rat H4IIE hepatoma cells

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Abstract

Phytochemical investigation of plants used in traditional Indonesian medicine (Jamu) yielded lignans (pinoresinol, 9α-hydroxypinoresinol and salicifoliol), flavonoids (3-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosylkaempferol, luteolin and apigenin) and coumarins (coumarin, 8-hydroxycoumarin and 5-hydroxycoumarin). The beneficial effects of the respective plants for human health are thought to be associated with antioxidative activity. In the present study, the antioxidative capacity of the isolated compounds was determined in an in-vitro assay. Luteolin and kaempferol (cleavage product of 3-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosylkaempferol, which is thought to be formed in the intestine) showed strong antioxidant activity; pinoresinol and 9α-hydroxypinoresinol showed only minor antioxidative effects. The coumarins, as well as apigenin and 3-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosylkaempferol were inactive. The antioxidative effects of luteolin, kaempferol and pinoresinol were further investigated in H4IIE rat hepatoma cells. A strong protective effect of kaempferol and luteolin was found against H_2O_2-mediated intracellular reactive oxygen species formation measured using the dichlorofluorescein assay and H_2O_2-mediated DNA strand breaks. Pinoresinol did not have a protective effect against H_2O_2-mediated DNA-damage, but in the dichlorofluorescein assay, an antioxidative effect was detectable. During studies with H4IIE cells, kaempferol, luteolin and pinoresinol were taken up by the cells within 60 min. The flavonoids were found to be relatively toxic at higher concentrations, while pinoresinol was less cytotoxic. In conclusion, kaempferol and luteolin, at low concentrations (≤ 50 μM), protect H4IIE cells against oxidative stress but are cytotoxic at higher concentrations; the biological effects of pinoresinol are less prominent in comparison. These results are important for the identification of pharmacologically active substances from traditional Indonesian medicinal plants.

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Introduction

Traditional Indonesian herbal medicine (Jamu) has been used since ancient times by large numbers of Indonesians. Around 75% of the 200 millions Indonesians consume various types of Jamu products on a regular basis to prevent or heal diseases. Hundreds of plant drugs are used for Jamu prescriptions, for example rhizomes such as ginger (*Zingiber officinale*), barks such as cinnamom (*Cinnamomum verum*), and fruits such as coriander (*Coriandrum sativum*) (Proksch 1997).

We investigated the antioxidative properties of several plants frequently used in Jamu: *Sonchus arvensis* L., Asteraceae, (Daun Tempuyung) and *Graptophyllum pictum* Griff., Acanthaceae, (Daun Ungu) had no activity, whereas *Alyxia reinwardtii* Bl., Apocynaceae, (Pulosari), *Guazuma ulmifolia* Lam., Sterculiaceae, (Daun Jati Belanda) and *Sauropus androgynus* Merr., Euphorbiaceae, (Daun Katuk) had antioxidative properties. We isolated several compounds from the latter species to identify the active principles that are responsible for the observed antioxidative effects of the extracts.

We focussed on antioxidative activity as reactive oxygen species (ROS) are thought to be an important factor in many human diseases such as Alzheimer's disease and

Parkinson's disease. Oxidative stress may also contribute to the pathology of ageing, inflammation and cancer development (Sayre et al 2001). The generation of ROS is a physiological process due to the oxidative metabolism of the cell. Usually, ROS are inactivated by different antioxidative mechanisms, for example antioxidative enzymes or low molecular antioxidants. If the generation of ROS exceeds the antioxidative potency, this leads to an accumulation of these molecules in the cell (hydroxyl radicals, superoxide anions, hydrogen peroxide) causing oxidative stress. This excess of highly reactive substances can result in severe damage to cellular macromolecules such as DNA, proteins and lipids. It is expected that oxidative stress is also responsible for molecular events leading to cancer formation as a result of, for example, the induction of oxidative DNA damage (mutations) and alterations in intracellular signal transduction (Allen & Tresini 2000; Martin & Barrett 2002).

Polyphenolic compounds, including flavonoids and lignans, have been shown to be potent antioxidants due to their radical scavenging activity and their ability to complex heavy metal ions, for example iron and copper, which are involved in Fenton-like reactions. Epidemiological studies have also suggested that polyphenols are associated with a reduced incidence of coronary heart disease (Hollman et al 1996).

Flavonoids occur ubiquitously in dietary products of plant origin and are predominantly present in a glycosidic form, although flavonoid aglycones are also occasionally detected (e.g. myricetin in red wine). The glycosides are converted by intestinal bacteria to the biologically active aglycones (Hollman & Katan 1998). Flavonoids possess a broad spectrum of biochemical and pharmacological activities (Formica & Regelson 1995). Lignans are of increasing interest because of their potential anticarcinogenic, oestrogenic and anti-oestrogenic activities and antioxidative effects (Adlercreutz 1995).

The present study aimed to provide data on the identification of pharmacologically active secondary metabolites from traditional Indonesian medicinal plants. Following the isolation of different compounds from plant extracts exhibiting antioxidative activity, the most active antioxidants were investigated for their uptake into rat H4IIE cells and antioxidative effects within this cellular system. To our knowledge, the antioxidative capacity of pinoresinol in a cellular system has not been previously examined.

Materials and Methods

Materials

Plant material was provided by PT Indofarma (Jakarta, Indonesia) where voucher specimens are on file. Silica gel (Kieselgel 60; Merck, Darmstadt, Germany) was used for vacuum liquid chromatography and column chromatography. Kaempferol was from Calbiochem (Bad Soden, Germany). All other chemicals were of analytical grade and were purchased from Sigma (Deisenhofen, Germany) or Merck.

Isolation of compounds

Isolation of luteolin and apigenin from G. ulmifolia
Dried leaves (760 g) were consecutively extracted with dichloromethane and methanol by Soxhlet. The dried methanolic extract was reconstituted in aqueous methanol (90:10), then extracted with *n*-hexane. The aqueous phase was taken to dryness, redissolved in water and subjected to liquid/liquid partitioning against ethyl acetate. The ethyl acetate phase was separated by vacuum liquid chromatography, yielding apigenin in the fraction eluted with *n*-hexane/ethyl acetate (35:65). The fraction obtained with *n*-hexane/ethyl acetate (10:90) was subjected to gel filtration over Sephadex LH-20 (methanol) to yield luteolin. Both compounds were identified by their online UV (P580A; Dionex, Idstein, Germany) and mass spectra (LCQ Deca; Thermo Finnigan, Dreieich, Germany) compared with authentic standards, as well as by ^{1}H-NMR (NMR spectrometer: DRX 500; Bruker, Rheinstetten, Germany) equipped with TBI (triple broadband inverse) gradient head for ^{1}H and HMBC spectra (500 MHz), and QNP (quadro nuclear probe) head for ^{13}C spectra (125 MHz).

Isolation of coumarins and lignans from A. reinwardtii
The dried bark (300 g) was extracted consecutively with ethyl acetate and methanol. The methanolic extract was evaporated, dissolved in water and extracted with ethyl acetate. The organic phase was taken to dryness, redissolved in methanol and extracted with *n*-hexane. After precipitation of the methanolic phase with water, the supernatant was loaded onto a silica gel column (*n*-hexane/ethyl acetate 30:70) yielding coumarin, 8-hydroxycoumarin, 5-hydroxycoumarin, pinoresinol ($[\alpha]^{20}_{D} + 56.7^{\circ}$, c 1.0 in acetone; 241 MC polarimeter; Perkin Elmer, Rodgau-Jügesheim, Germany), 9α-hydroxypinoresinol ($[\alpha]^{20}_{D} + 35.0^{\circ}$, c 0.46 in methanol), and salicifoliol ($[\alpha]^{20}_{D} + 55.0^{\circ}$, c 0.24 in methanol). Pinoresinol was applied onto a chiral high performance liquid chromatography (HPLC) column (Chiracel OD-H $250 \times 4.6\,\text{mm} \times 5\,\mu\text{m}$; eluent: ethanol and *n*-hexane; gradient: 50–60% ethanol in 45 min). The chromatogram showed two peaks, (−)-pinoresinol and (+)-pinoresinol, which were identified by co-injection of respective standards. The ratio of the peak areas of (−)-pinoresinol to (+)-pinoresinol was 1:9.

Isolation of a kaempferoldiglucoside from S. androgynus
The leaves (300 g) were extracted in the same way as described for the bark of *A. reinwardtii*. After vacuum liquid chromatography of the methanolic extract over silica gel, the fraction eluted at 100% methanol was chromatographed over a Sephadex LH-20 column using methanol as eluent, from which 3-O-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosylkaempferol ($[\alpha]^{20}_{D} - 5.6^{\circ}$, c 0.5 in methanol) was isolated. The identity of the sugar moieties was confirmed by gas liquid chromatography analysis following hydrolysis as described previously (Gerwig et al 1977).

Antioxidative capacity

For the qualitative analysis, an aliquot of the plant extracts was applied onto a thin layer chromatography plate

(Kieselgel 60 F_{254}) and developed in dichloromethane/methanol 95:5. Eluted compounds with antioxidant activity were detected by spraying the thin layer chromatography plates with 2,2-diphenyl-1-picrylhydrazyl (DPPH) reagent (1%) (Kirby & Schmidt 1997). The antioxidant activity was quantified by the decrease in absorption of each of the isolated compounds in 0.0045% DPPH solution (final concentration of the sample in the cuvette was 76 μM) monitored at 517 nm using a spectrophotometer (Li et al 1999). The absorbance of DPPH in methanol (with or without compounds) was measured after 2 min. The antioxidant capacity of each compound was measured in relation to propyl gallate (positive control) set as 100% antioxidative activity and vehicle (dimethylsulfoxide) set as 0% antioxidative capacity.

Cell culture

H4IIE rat hepatoma cells were grown in Dulbecco's modified Eagle's medium containing 4.5 g L^{-1} glucose and 2 mM L-glutamine, 10% fetal bovine serum, 100 units mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. The cells were maintained in a humidified atmosphere at 37°C with 5% CO_2.

Cellular uptake

A total of 10^6 cells were plated in 100-mm cell culture dishes, allowed to attach for 48 h, and then incubated with polyphenols (100 μM). Cells were washed three times (phosphate-buffered saline), taken up in methanol and lysed by sonification. After freezing, the lysate was shaken (5 h) and the supernatant was injected onto a HPLC system (Dionex). The separation column (125 × 4 mm) was prefilled with Eurosphere 100 C-18 (5 μm) (Knauer, Berlin-Zehlendorf, Germany): the HPLC gradient was 10–100% methanol/water adjusted to pH 2 with *o*-phosphoric acid. The substances that were taken up were identified by their online UV spectra and by comparison with authentic standards. The peak area at 254 nm was recorded and compounds were quantified via an external calibration curve. Experiments were performed in triplicate.

Determination of DNA strand breaks (single cell gel electrophoresis)

A total of 500 000 cells were seeded in a 35-mm cell culture dish and incubated 24 h later with polyphenols (50 μM, 1 h), followed by an incubation with 500 μM H_2O_2 for 2 h. Then, cells were suspended and an aliquot of the suspension was added to 1% low melting point agarose and pipetted onto a frosted glass microscope slide precoated with 1% normal melting point agarose. The slides were incubated in ice-cold lysis solution and then electrophoresis was performed. The slides were neutralized, stained with ethidium bromide and analysed using a Zeiss Axiolab fluorescence microscope (Zeiss filter set 15). The average image length of 50 randomly selected cells was counted (Singh et al 1988).

Intracellular 2′,7′-dichlorofluorescein (DCF) formation

A total of 50 000 cells/well were seeded on a 96-well microtitre plate, allowed to attach for 24 h, and then the medium was changed (without phenol red). Cells were first incubated with different polyphenols (10 μM) for 60 min, then 50 μM H_2DCF diacetate was added (30 min). The oxidation of intracellular non-fluorescent H_2DCF to highly fluorescent DCF was measured after addition of H_2O_2 (500 μM) (excitation: 485 nm; emission: 525 nm).

Determination of cytotoxicity

A total of 10 000 cells/well were plated in 96-multiwell dishes, allowed to attach for 24 h, and then treated with polyphenols (24 h). The cells were incubated for 3 h with 16 μg mL^{-1} neutral red in the medium (Babich et al 1986). Medium was removed and the cells rinsed with phosphate-buffered saline. The cells were then fixed on the plate with 1% formaldehyde and lysed using 50% ethanol, 1% acetic acid. The concentration of neutral red was measured photometrically at 560 nm.

Statistical analysis

All data are given as mean ± s.e.m. The significance of changes in the test responses was assessed using analysis of variance. Differences were considered significant at $P < 0.05$.

Results

A. reinwardtii yielded coumarin, 8-hydroxycoumarin, 5-hydroxycoumarin, pinoresinol, 9α-hydroxypinoresinol and salicifoliol. *G. ulmifolia* yielded apigenin and luteolin. *S. androgynus* yielded 3-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosylkaempferol. The structures of the compounds are given in Figure 1. This is the first report on the occurrence of 9α-hydroxypinoresinol and salicifoliol in *A. reinwardtii*. Apigenin and luteolin are also reported in *G. ulmifolia* for the fist time.

All compounds were unambiguously identified by NMR spectroscopy and mass spectrometry, and by comparison with published data (Sadavongvivad & Supavilai 1977; Abe & Yamauchi 1988; Gonzalez et al 1989; Schmidt 1994; Wang & Lee 1997).

The lignans pinoresinol and 9α-hydroxypinoresinol showed only minor antioxidative effects. The flavonoids luteolin and kaempferol, the aglycone of 3-*O*-β-D-glucopyranosyl-(1→ 6)-β-D-glucopyranosylkaempferol all exhibited strong antioxidative activity that was comparable with propyl gallate. On the other hand, 3-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosylkaempferol itself, apigenin and the isolated coumarins had no antioxidative activity (Figure 2).

Luteolin and kaempferol (at 100 μM) were rapidly taken up by the cells within the first 30 min, reaching

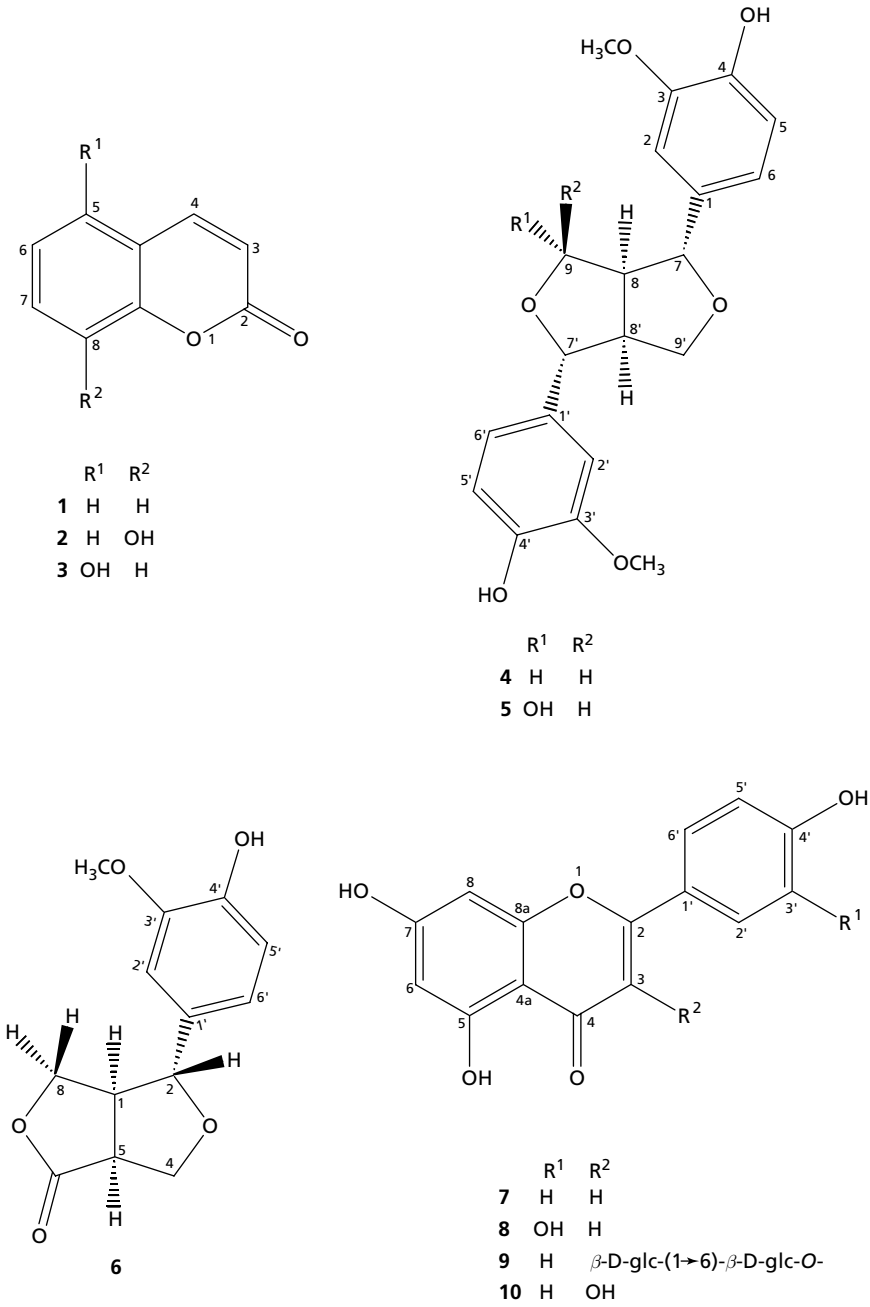

Figure 1 Structures of isolated compounds. Coumarins: **1**, coumarin; **2**, 8-hydroxycoumarin; **3**, 5-hydroxycoumarin. Lignans: **4**, pinoresinol; **5**, 9α-hydroxypinoresinol; **6**, salicifoliol. Flavonoids: **7**, apigenin; **8**, luteolin; **9**, 3-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosylkaempferol; **10**, kaempferol.

maximal intracellular concentrations of $1.68 \pm 1\,\text{nmol}/10^6$ cells (30 min) and $6.24 \pm 3.8\,\text{nmol}/10^6$ cells (30 min), respectively. The concentration of the intracellular flavonoids then declined within the next 3 h (0.02 and $0.07\,\text{nmol}/10^6$ cells, respectively), presumably due to metabolic degradation. Pinoresinol was taken up to a minor extent, reaching a maximal intracellular concentration of only $0.76 \pm 0.56\,\text{nmol}/10^6$ cells after 60 min, and then declining to $0.164\,\text{nmol}/10^6$ cells after 4 h. As expected, 3-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosylkaempferol was not taken up by the cells.

Incubation with H_2O_2 led to an intracellular formation of ROS, measured as oxidation of non-fluorescent H_2DCF to highly fluorescent DCF. If the cells were preincubated with 10 μM of kaempferol or luteolin (60 min), the increase in H_2O_2-mediated DCF formation was strongly inhibited, while the antioxidative effect of pinoresinol was not as prominent in comparison (Figure 3).

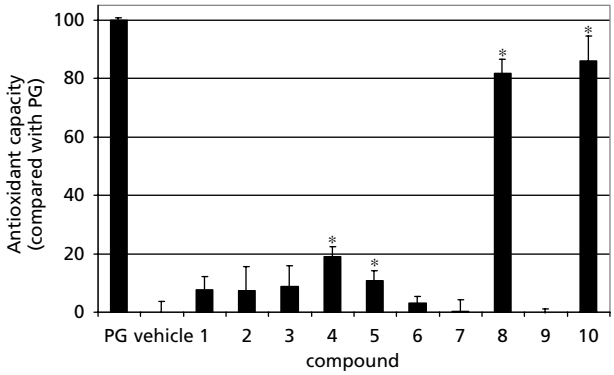

Figure 2 Antioxidative capacity in-vitro (DPPH assay). The antioxidant capacity was measured in relation to the reference substance propyl gallate (PG) set as 100% antioxidative activity and vehicle (dimethylsulfoxide) set as 0% antioxidative capacity (means ± s.d., n = 3, $*P < 0.05$). The numbers on the x-axis represent the compounds as given in Figure 1.

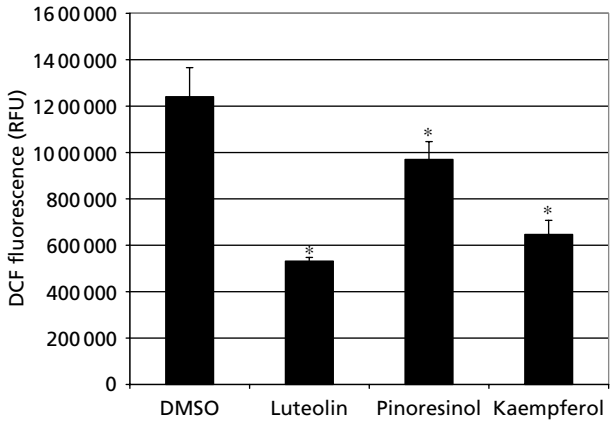

Figure 3 Protective effect on H_2O_2-mediated DCF formation. H4IIE cells were pre-incubated with 10 μM kaempferol, luteolin or pinoresinol for 1 h. After administration of the fluorescence probe H_2DCF/DA (50 μM, 0.5 h), ROS formation was measured using a Wallac Victor^2 1420 fluorescence reader. After 60 min the cells were incubated with 1 mM H_2O_2 and protective effects of kaempferol, luteolin and pinoresinol on H_2O_2-mediated DCF-formation were analysed (DCF fluorescence after 60 min ± s.e.m., n = 3, $P < 0.05$).

Oxidative stress led to a dose-dependent increase of DNA strand breaks in H4IIE cells. The comet assay showed an increase in average image length (nucleus plus comet tail) from 12.56 ± 1.8 μm (control) to 40.8 ± 4.9 μm (500 μM H_2O_2, 2 h). Pre-incubation of cells with 50 μM of luteolin or kaempferol (1 h) decreased the H_2O_2-mediated DNA strand break formation by approximately 70–80%; incubation with pinoresinol caused no significant reduction (Figure 4).

Incubation of H4IIE cells with kaempferol, luteolin or pinoresinol (100 μM) had a negative effect on cell viability. Incubation with kaempferol or luteolin led to a decrease of viable cells to approximately 40%. Pinoresinol was less toxic in comparison, leading to approximately 25% cell death (Figure 5). The EC50 (effective concentration 50%) values of kaempferol, luteolin and pinoresinol were 49, 92 and 215 μM, respectively. Apigenin and 3-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosylkaempferol were not tested for cytotoxicity as both compounds were inactive in the DPPH assay.

Discussion

Apigenin and luteolin were isolated from the leaves of *G. ulmifolia*, which are traditionally used to prepare slimming teas. The antioxidative activity of luteolin can be explained by its catechol moiety present in the B ring, which is essential for the radical scavenging properties, while apigenin, which has only one hydroxy group on the B ring, was inactive (Cotelle et al 1996). Luteolin was taken up by H4IIE cells and showed strong activity in the DCF formation assay as well as a protective effect against H_2O_2-induced DNA strand breaks. The protective effect of luteolin against H_2O_2-mediated DNA strand breaks in H4IIE cells is in agreement with results previously obtained with human lymphocytes (Noroozi et al 1998). Luteolin has been shown to contribute to the antioxidant activity of artichoke leaf extracts on ROS in human leucocytes (Brown & Rice-Evans 1998). An antioxidative effect of luteolin in cultured retinal cells has been demonstrated by measuring a decrease in malondialdehyde and DCF formation (Areias et al 2001). The toxicity of luteolin in H4IIE cells was in the same order of magnitude as in MiaPaCa-2 cancer cells (Lee et al 2002).

It is interesting to note that the presence of apigenin and luteolin in air-dried leaves of *G. ulmifolia*, which are used for Jamu preparations, probably results from post-harvesting degradation of glycosidic precursors. Analysis of fresh plant material indicated only the presence of (so far unidentified) apigenin and luteolin glycosides but gave no indication of the occurrence of free apigenin or luteolin in intact plant tissue (Steffan, B., Proksch, P., unpublished results). Similar phenomena were reported for dried versus fresh chamomile flowers (Schreiber et al 1990).

Coumarin, 5-hydroxycoumarin and 8-hydroxycoumarin, as well as the lignans pinoresinol, 9α-hydroxypinoresinol and salicifoliol were isolated from the bark of *A. reinwardtii*, which is used against influenza, cough and fever. Pinoresinol was isolated in racemic form with the (+)-enantiomer dominating over the (−)-enantiomer at a ratio of 9:1. In the DPPH assay, only pinoresinol and 9α-hydroxypinoresinol showed weak antioxidative activity. Pinoresinol was taken up by H4IIE cells, showing a weak activity in the DCF formation assay and no protection against H_2O_2-induced DNA strand breaks, probably due to its weaker antioxidative activity as compared with the flavonoids investigated in this study. Pinoresinol was also found to be only slightly cytotoxic. In the literature, the biological effects of lignans are controversially discussed because of their chemically heterogeneous structures. The mammalian lignans enterolactone and enterodiol were found to be ineffective in preventing H_2O_2-induced DNA

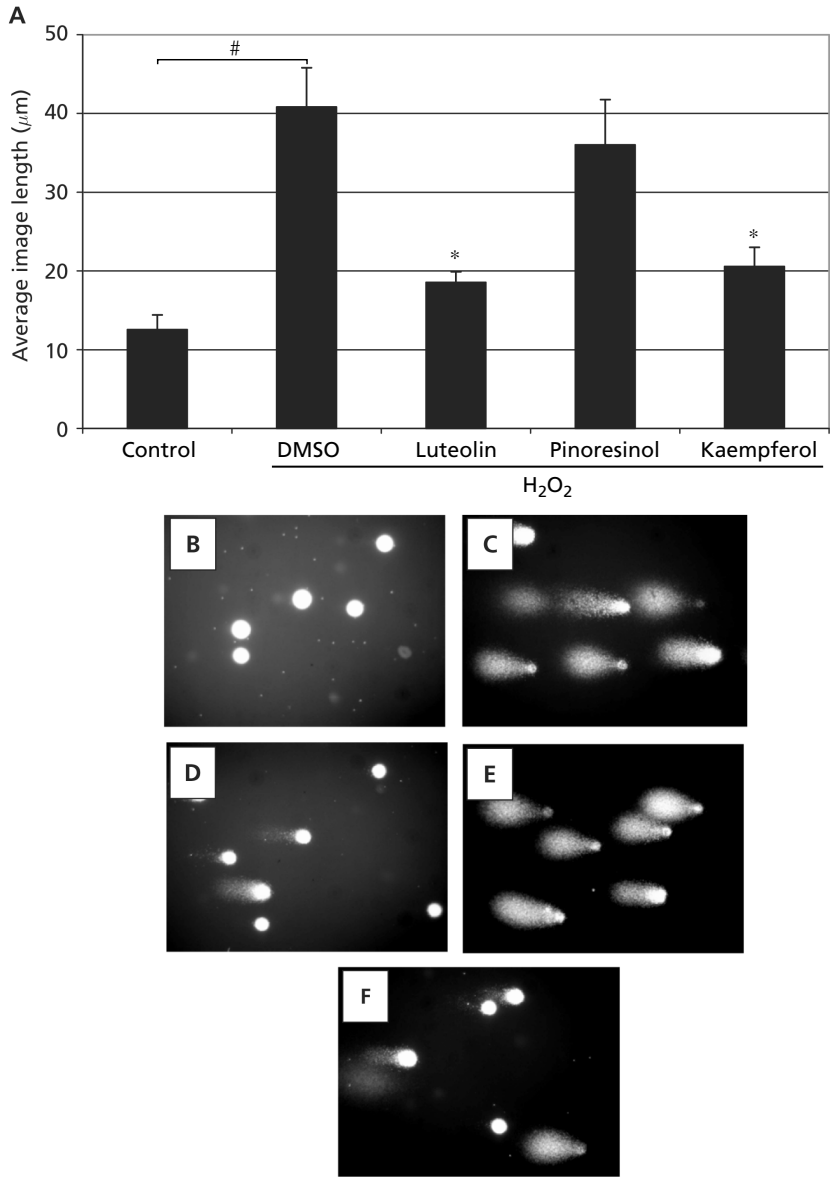

Figure 4 Protective effect on H_2O_2-mediated DNA strand break formation. To analyse the protective effects of kaempferol, luteolin and pinoresinol, cells were pre-incubated with these compounds (50 μM) for 1 h followed by an incubation with 500 μM H_2O_2 for 2 h. DNA strand breaks were analysed by single cell gel electrophoresis (comet assay). A. The average image length (μm) shown represents the extent of H_2O_2-mediated DNA strand breaks with or without the compounds (means $\pm$ s.d., n = 3, $P < 0.05$). Representative images of the decrease in H_2O_2-mediated DNA strand break formation are also shown. B. Control cells. C. H_2O_2-induced comet formation (500 μM, 2 h). D. Pre-incubation (1 h) with 50 μM luteolin followed by 500 μM H_2O_2 for 2 h. E. Pre-incubation (1 h) with 50 μM pinoresinol followed by 500 μM H_2O_2 for 2 h. F. Pre-incubation (1 h) with 50 μM kaempferol followed by 500 μM H_2O_2 for 2 h.

damage in HT-29 cells (Pool-Zobel et al 2000), while the plant-derived lignan nordihydroguaiaretic acid (but not enterolactone and enterodiol) exhibited potent antioxidant activity in HepG2 cells as determined by inhibition of H_2DCF oxidation (Harper et al 1999).

The leaves of *S. androgynus* are used traditionally to increase lactation after childbirth. The major phenolic constituent 3-*O*-β-D-glucopyranosyl-(1$\rightarrow$6)-β-D-glucopyranosylkaempferol as well as the aglycone kaempferol were subjected to a DPPH assay. The aglycone showed strong antioxidant properties, whereas the glycoside had no activity when measured at equimolar concentrations. The presence of either a catechol or pyrogallol type B ring or of a hydroxy substituent in position 3 of the C ring was found to be important for radical scavenging properties (Cotelle et al 1996). In the case of 3-*O*-β-D-glucopyranosyl-(1$\rightarrow$6)-β-D-glucopyranosylkaempferol the OH group at C-3 is substituted by a sugar unit, resulting in loss of activity. Kaempferol was not able to protect against oxidative DNA damage in Caco-2 cells (Duthie & Dobson 1999). In contrast to these results, we found that kaempferol is rapidly taken up by the H4IIE cells, resulting in a strong

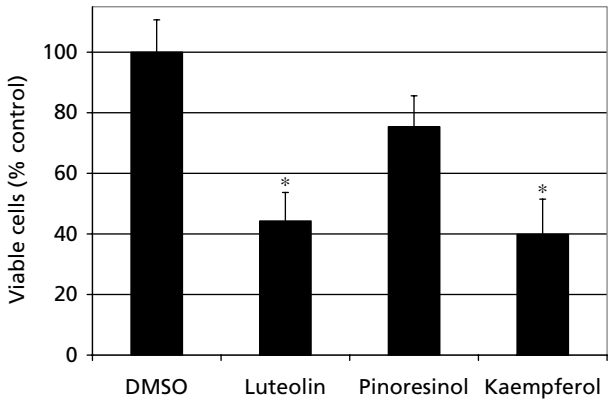

Figure 5 Cytotoxicity. H4IIE cells were incubated with kaempferol, luteolin or pinoresinol (100 μM) for 20 h, then neutral red accumulation was measured for 3 h. Results are expressed as a percentage of the control value (means ± s.e.m., n = 4, $P < 0.05$).

decrease of H_2O_2-mediated DNA strand breaks as well as H_2O_2-mediated DCF formation. However, the viability of H4IIE cells was decreased after incubation with kaempferol (100 μM: 36% viable cells), comparable with HT-29 cells (136 μM: 50% viable cells; Kuntz et al 1999).

Conclusion

The beneficial effects of plants used in traditional Indonesian herbal medicine (Jamu) are, among other factors, thought to be associated with antioxidative activity. We isolated compounds showing antioxidative activity, tested them in an in-vitro assay (DPPH assay) and determined their protective effects in a cellular system (rat H4IIE hepatoma cells). Kaempferol (resulting from cleavage of 3-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosylkaempferol in the intestine), luteolin and pinoresinol were rapidly taken up by the cells. Kaempferol and luteolin protected H4IIE cells against oxidative stress at low concentrations (≤50 μM) but were cytotoxic at higher concentrations (100 μM), while pinoresinol had only minor antioxidative effects. The ability of kaempferol and luteolin to inhibit oxidative DNA strand breaks as investigated using the comet assay and DCF formation supports their suggested role as protective agents against diseases such as cancer. These results are important for the identification of pharmacologically active substances in traditional Indonesian medicine considered for disease prevention.

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