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Evaluation of the antioxidant activity of *Ruellia tuberosa*

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Abstract

The antioxidant activity of *Ruellia tuberosa* L. (Acanthaceae) was investigated by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical-scavenging assay and the hydrogen peroxide-induced luminol chemiluminescence assay. The methanolic extract (ME) and its four fractions of water (WtF), ethyl acetate (EaF), chloroform (CfF), and *n*-hexane (HxF) were prepared and then subjected to antioxidant evaluation. The results of both methods revealed that *R. tuberosa* possesses potent antioxidant activity. The antioxidant activities of the different fractions tested decreased in the order of EaF > CfF > ME > WtF > HxF according to the hydrogen peroxide-induced luminol chemiluminescence assay, and results were the same with the exception of the rank order of HxF and WtF according to the DPPH free radical-scavenging assay. The results provide useful information on the pharmacological activities associated with free radicals of this traditional folk remedy.

Keywords: *Ruellia tuberosa*; Antioxidant activity; 1,1-Diphenyl-2-picryl-hydrazyl (DPPH); Chemiluminescence

1. Introduction

Ruellia tuberosa L., a tropical perennial plant, belongs to the family Acanthaceae and is distributed in Southeast Asia, including Thailand and Laos. *R. tuberosa* was introduced into Taiwan many years ago as a folk medicine due to its diuretic, diabetic, antipyretic, analgesic, and antihypertensive properties (Chiu & Chang, 1995). Recently, it is also being used as one of the components in a herbal drink in Taiwan. However, relatively little information has been obtained on its pharmacological activities.

Free radicals provoked by various environmental chemicals as well as endogenous metabolism are involved in a number of diseases like tumors, inflammation, shock, atherosclerosis, diabetes, infertility, gastric mucosal injury, and ischemia due to the oxidative damage to DNA, lipids, and proteins and which can result in failure of cellular functions (Jin & Chen, 1998; Kasai, Fukada, Yamaizumi, Sugie, & Mori, 2000; Sharma & Agarwal, 1996; Yesilada, Tsuchiya, Takaishi, & Kawazoe, 2000; Wallace, 1997). Consumption of antioxidants from plant materials that inhibit free radical formation or accelerate their elimination has been associated with a lowered incidence of these diseases as a consequence of alleviating the oxidative stress of free radicals (Ames, 1983; Leong & Shui, 2002); accordingly, antioxidants have recently garnered increased research interest. In view of the number of diseases associated with free radical generation, the present study attempted to estimate the antioxidant activity of *R. tuberosa* by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical-scavenging assay and the hydrogen peroxide-induced luminol chemiluminescence assay.

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2. Materials and methods

2.1. Materials

Stems of *R. tuberosa* were collected from Daren E. Road, Pingtung, southern Taiwan in November 2003. Botanical identification was confirmed by Mr. Kao, Herbarium, Department of Botany, National Taiwan University, Taipei, Taiwan. Vitamin C (ascorbic acid), luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), and DPPH (2,2-diphenyl-1-picrylhydrazyl) were purchased form Sigma Chemicals (St. Louis, MO, USA). All other chemicals were of analytical reagent grade.

2.2. Preparation of extracts of R. tuberosa

Dried stems of *R. tuberosa* (500 g) were cut up and subjected to extraction by macerating them in methanol for 10 days at room temperature in a dark cabinet. After solvent evaporation in a rotary evaporator (Büchi, Flawil, Switzerland), the methanolic extract (ME) was further fractionated through solvent–solvent partitioning to obtain different fractions according to the operation flowchart given in Fig. 1. The four solvents used to achieve high to low polarity for solvent–solvent partitioning were water, ethyl acetate, chloroform, and *n*-hexane. The ME and its four fractions of water (WtF), ethyl acetate (EaF), chloroform (CfF) and *n*-hexane (HxF) were stored in an electronic dry cabinet protected from light with aluminum foil (Komry, Taipei, Taiwan) after solvent evaporation. The ME and its four fractions were then subjected to antioxidant analysis.

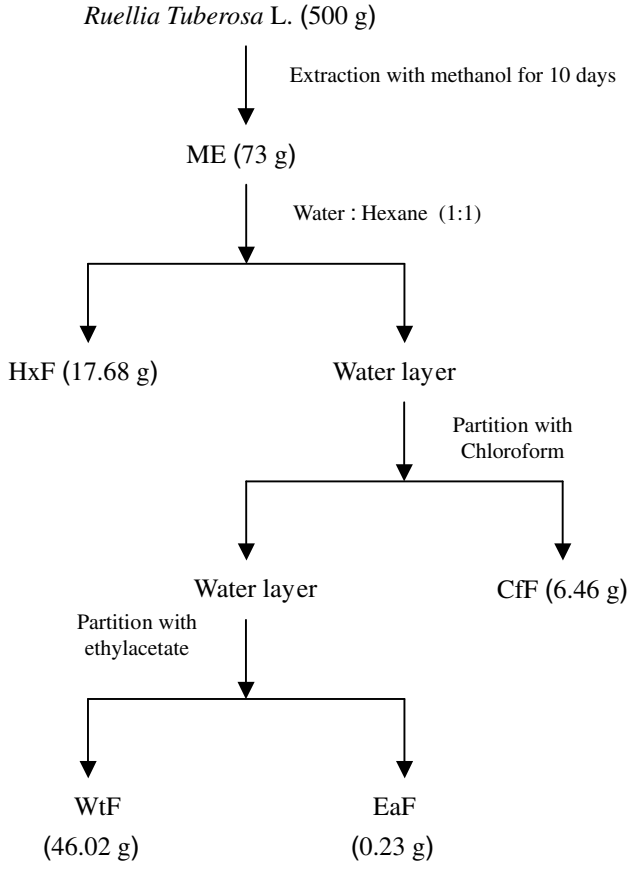

Fig. 1. Preparation flowchart of the methanolic extract (ME) of *Ruellia tuberosa* and its fractions.

2.3. DPPH free radical-scavenging assay

The free radical-scavenging activity of ME and its fractions on the DPPH radical was assessed using the method described by Fenglin, Ruili, Bao, and Liang (2004) with some modifications. A stock solution (1 mg/ml) of each extract was prepared and diluted with methanol to various concentrations. An aliquot of 50 μl of each dilution was transferred into a 96-well microplate (NUNC, Roskilde, Denmark). A working solution of DPPH (250 μM) in methanol was freshly prepared and then an aliquot of 150 μl was added to each well. After incubation for 30 min, the quenching at an absorbance of 490 nm was measured on an ELISA reader (ThermoLabsystems, Cheshire, UK). Each dilution was performed at least in triplicate. Free radical-scavenging activities of test samples and the positive control (Vit. C) were expressed in terms of IC_{50} values, which is the concentration of a sample required to decrease the absorbance at 490 nm by 50% compared to the control response.

2.4. Hydrogen peroxide-induced luminol chemiluminescence assay

The hydrogen peroxide-induced luminol chemiluminescence (CL) assay was performed as described by Sun et al. (1998). CL measurements were carried out using a CL analyzer (CLA-2100, Tohoku, Japan). A luminol solution (5 μg/ml, in PBS buffer at pH 7.4) and a hydrogen peroxide solution (30 mM, in distilled water) were freshly prepared before the experiment. For each measurement, a mixture of 0.1 ml of the luminol solution and 0.2 ml of the sample was added to a special chamber unit (Model TLU-21) including a stainless steel cell with a magnetic stirrer and stirrer bar, in an absolutely dark chamber of the CL analyzing system (model CLA-2100, Tohoku Electronic Industrial, Sendai, Japan). This system contains a photon detector (model CLA-2100), a CL counter (model CLC-10), a water circulator (model CH-20), and a Pentium 4 HP personal computer system. The cooler circulator was connected to the model CLA-2100 photon detector to maintain the temperature at 5 °C. After 120 s, an aliquot (0.1 ml) of the hydrogen peroxide solution was injected into the special chamber unit of the CL analyzer. Total CL counts were continuously recorded for 300 s. Each measurement was performed at least in triplicate. The

antioxidant activities of the test samples and the positive controls (Vit. C) were expressed in terms of IC_{50} values, which is the concentration needed to decrease hydrogen peroxide-induced CL by 50% in comparison to the control response.

3. Results and discussion

3.1. Preparation of methanolic extracts of R. tuberosa and its fractions

Due to the complicated constituents and pharmacological diversities of plants, in vitro bioassay-guided fractionation has been effectively applied to screen the biological activities that contribute important indications for investigating the characteristics of active components (Garg, Talwar, & Upadhyay, 1998; Yesilada et al., 2000). As shown in Fig. 1, the methanolic extract (ME) of *R. tuberosa* was fractionated through solvent–solvent partitioning to obtain four fractions of water (WtF), ethyl acetate (EaF), chloroform (CfF), and *n*-hexane (HxF). The recoveries of WtF, EaF, CfF, and HxF were about 63.0%, 0.3%, 8.9%, and 24.2%, respectively, indicating that the constituents of *R. tuberosa* belong mainly to the two opposing extremes of polarity.

3.2. DPPH free radical-scavenging assay

The DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical is a stable free radical, which has been widely accepted as a tool for estimating free radical-scavenging activities of antioxidants (Fenglin et al., 2004; Jin & Chen, 1998; Kim, Guo, & Packer, 2002; Leong & Shui, 2002). The percentages of DPPH remaining in the presence of the ME and its fractions at different concentrations are shown in Fig. 2. The proportions of the DPPH remaining with the ME and WtF slightly decreased; great decreases in a concentration-dependent manner of DPPH remaining were found in EaF and

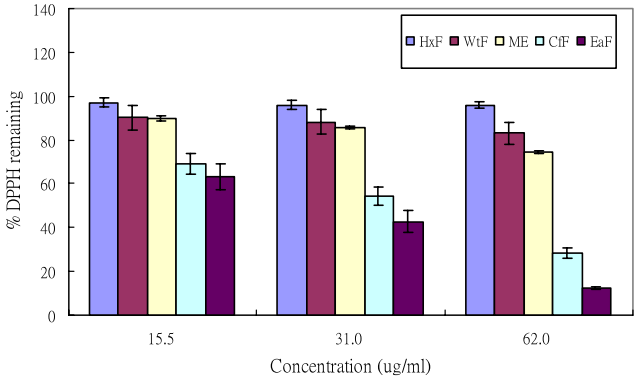

Fig. 2. DPPH remaining after addition of the methanolic extract (ME) of *Ruellia tuberosa* and its fractions for 30 min. Data are presented as means ± standard deviation ($n \geqslant 3$).

Table 1
Antioxidant activities of the methanolic extract (ME) of *Ruellia tuberosa* and its fractions using the DPPH free radical-scavenging assay and hydrogen peroxide-induced luminol chemiluminescence (CL) assay

Samples	$IC_{50/DPPH}$ (μg/ml)[a]	$IC_{50/CL}$(μg/ml)[b]
ME	228 ± 1	10.2 ± 2.6
HxF	2208 ± 8	12.1 ± 0.9
CfF	34.8 ± 0.8	7.2 ± 0.9
EaF	28.6 ± 0.7	2.8 ± 0.6
WtF	429 ± 14	13.6 ± 1.6
Vit. C	6.4 ± 0.4	18.6 ± 2.2

Data are presented as the mean standard deviation ($n \geqslant 3$).

[a] The antioxidant activity was evaluated as the concentration of the test sample required to decrease the absorbance at 490 nm by 50% in comparison to the control response.

[b] The antioxidant activity was evaluated as the concentration of the test sample needed to decrease the hydrogen peroxide-induced CL by 50% in comparison to the control response.

CfF indicating that *R. tuberosa* possesses potent free radical-scavenging activity with the exception of HxF. By comparing ME and its active fractions, the free radical-scavenging activities decreased in the order of EaF > CfF > ME > WtF. The free radical-scavenging activity of the ME was less than those of EaF and CfF, which resulted from increasing the active components/units through condensation effects during the solvent–solvent partitioning processes. In addition, the results also indicated that the active components existed mainly in the medium-polar fractions, EaF and CfF. In order to quantify the antioxidant activity, the IC_{50}, which is the concentration of sample required to decrease the absorbance at 490 nm by 50%, was further calculated and is shown in Table 1. The lower the IC_{50} value is, the greater the free radical-scavenging activity is. By comparing the IC_{50} value of the ME and those of its active fractions with that of an authentic antioxidant, Vit. C, we found that the antioxidant activity of EaF was less than that of Vit. C. There is limited literature available on investigations of the constituents except for a report published about 30 years ago, which found that *R. tuberosa* contained a flavonoid glycoside, apigenin-7-D-glucuronide (Wagner et al., 1971). Flavonoids are well-known antioxidant constituents in plants (Fenglin et al., 2004; Luximon-Ramma, Bahorun, Soobrattee, & Aruoma, 2002). A similar report found that *Spartium junceum*, a Turkish folk medicine, contained flavonoid glycosides, which possessed potent antioxidant activity by activity-guided fractionation (Yesilada et al., 2000). Accordingly, the antioxidant activity of *R. tuberosa* may be related to its flavonoid constituents.

3.3. Hydrogen peroxide-induced luminol chemiluminescence assay

The hydrogen peroxide-induced luminol chemiluminescence assay is another widely used method to

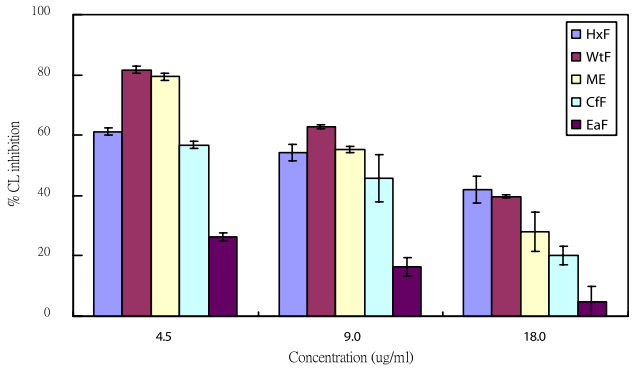

Fig. 3. Inhibitory activity on hydrogen peroxide-induced chemiluminescence after addition of the methanolic extract (ME) of *Ruellia tuberosa* and its fractions. Data are presented as means ± standard deviation ($n \geqslant 3$).

evaluate antioxidant activities (Desmarchelier, Romão, Coussio, & Ciccia, 1999; Murr et al., 1996; Navas & Jiménez, 1996). Chemiluminescence (CL) is the emission of light through an oxidizing reaction of luminol by hydrogen peroxide, a reactive oxygen species (ROS) and an intermediate during endogenous oxidative processes; CL formation in the presence of luminol can be applied to estimate the hydrogen peroxide elimination activity of antioxidants. The inhibition of hydrogen peroxide-induced CL in the presence of the ME or its fractions at different concentrations is shown in Fig. 3. The ME and its fractions could effectively inhibit CL formation in a concentration-dependent manner, indicating that *R. tuberosa* possesses antioxidant activity. By comparing the IC_{50} results between these two methods, the antioxidant activities decreased in the order of EaF > CfF > ME > WtF > HxF according to the hydrogen peroxide-induced luminol chemiluminescence assay, and these were the same with the exception of the rank order of HxF and WtF according to the DPPH free radical-scavenging assay as shown in Table 1. In addition, in a comparison of the IC_{50} values of the ME and its active fractions with Vit. C, the antioxidant activities of all extracts were greater than that of Vit. C. These differences indicated that the particular free radical species detected, as well as the reaction mechanism, affected the results, which are also similar to those reported by Mantle et al. (1998) and Parejo, Codina, Petrakis, and Kefalas (2000). In order to estimate correlations of results from these two methods, IC_{50} values from the DPPH free radical-scavenging assay were plotted against those from the hydrogen peroxide-induced luminol chemiluminescence assay. As shown in Fig. 4, a good correlation was observed ($r > 0.848$) between these two methods.

In a comparison of methods used in this study, both the DPPH free radical-scavenging assay and the hydrogen peroxide-induced luminol chemiluminescence assay showed the capability to determine the antioxidant activity of reference materials and samples. The hydrogen peroxide-induced luminol chemiluminescence assay was found to be more sensitive because the concentration measured was one order of magnitude less than the DPPH free radical-scavenging assay, which is in agreement with that reported by Parejo et al. (2000). Also, the lower sensitivity of the DPPH free radical-scavenging assay may have been due to the higher stability of DPPH with which more antioxidants need to react. Furthermore, determination of the antioxidant activity using the DPPH free radical-scavenging assay seems to barely represent the biological situation, because DPPH is an exogenous radical. In the case of the hydrogen peroxide-induced luminol chemiluminescence assay, hydrogen peroxide, an intermediate during endogenous oxidative metabolism and which mediates oxygen radical formation such as ·OH, may be used to predict the scavenging capability of antioxidants in biological systems. Nevertheless, both methods are capable of prescreening antioxidant activities.

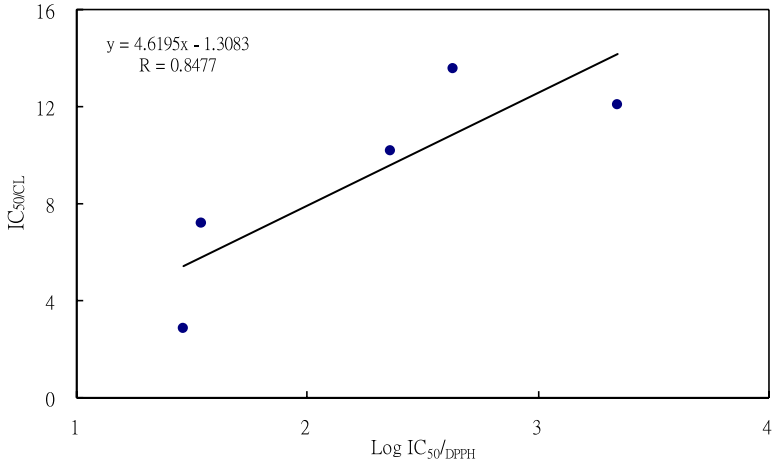

Fig. 4. Correlation of the antioxidant activities of the methanolic extract (ME) of *Ruellia tuberosa* and its fractions measured by the DPPH free radical-scavenging assay and the hydrogen peroxide-induced luminol chemiluminescence (CL) assay.

In conclusion, we demonstrate that *R. tuberosa* possesses potent antioxidant activity by the DPPH free radical-scavenging assay and hydrogen peroxide-induced luminol chemiluminescence assay. From the data obtained in these two methods, the antioxidant activities decreased in the order of EaF > CfF > ME > WtF > HxF according to the hydrogen peroxide-induced luminol chemiluminescence assay, which were the same with the exception of the rank order of HxF and WtF obtained from the DPPH free radical-scavenging assay. The antioxidant activity of *R. tuberosa* may be related to its flavonoid constituents. The results provide useful information on pharmacological activities associated with free radicals of this traditional folk remedy. Further studies are in progress to determine the antioxidant activity in vivo and to identify the active compounds present in this plant. From a health point of view, *R. tuberosa* is a meaningful component of herbal drinks due to its antioxidant activity.

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