Antioxidative and free radical scavenging effects of ecdysteroids from *Serratula strangulata*

Yu-Jun Cai, Jin-Qiu Dai, Jian-Guo Fang, Lan-Ping Ma, Li-Fen Hou, Li Yang, and Zhong-Li Liu

Abstract: The antioxidative and free radical scavenging effects of four ecdysteroids, 20-hydroxyecdysone (E1), 25-deoxy-11,20-dihydroxyecdysone (E2), 24-(2-hydroxyethyl)-20-hydroxyecdysone (E3), and 20-hydroxyecdysone-20,22-monoacetonide (E4), isolated from the Chinese herb *Serratula strangulata* have been investigated in vitro. These ecdysteroids could protect human erythrocytes against oxidative hemolysis induced by a water-soluble azo initiator 2,2′-azobis(2-amidinopropane hydrochloride) (AAPH). They could also inhibit the peroxidation of rat liver microsomes induced by hydroxyl radicals, as monitored by the formation of thiobarbituric acid reactive substances (TBARS), and prevent radical-induced decrease of membrane fluidity as determined by fluorescence polarization. They reacted with galvinoxyl radicals in homogeneous solution, and the pseudo-first-order rate constants were determined spectrophotometrically by following the disappearance of galvinoxyl radicals. Compounds E1 and (or) E3 were the most active in both antioxidative and radical-scavenging reactions.

Key words: ecdysteroid, *Serratula strangulata*, free radical, erythrocyte, lipid peroxidation, antioxidant.

Résumé : On a examiné in vitro les effets antioxydatifs et antiradicalaires de quatre ecdystéroïdes, 20-hydroxyecdysone (E1), 25-désoxy-11,20-dihydroxyecdysone (E2), 24-(2-hydroxyéthyl)-20-hydroxyecdysone (E3) et 20-hydroxyecdysone-20,22-monoacétonide (E4), isolés de la plante chinoise *Serratula strangulata*. Ces ecdystéroïdes pourraient protéger les érythrocytes humains contre l'hémolyse oxydative induite par l'initiateur azo hydrosoluble, chlorhydrate de 2,2′-azobis(2-amidinopropane) (AAPH). Ils pourraient aussi inhiber la peroxydation induite par les radicaux hydroxyl des microsomes hépatiques de rats, tel qu'indiqué par la formation de substances réagissant avec l'acide thiobarbiturique (TBARS), et prévenir la diminution induite par les radicaux de la fluidité membranaire, tel que déterminé en utilisant la polarisation de fluorescence. Ils ont réagi avec le radical galvinoxyl dans une solution homogène, et les constantes de pseudo-premier ordre ont été déterminées par spectrophotométrie en suivant la disparition du radical. Les composés E1 et (ou) E3 sont les plus actifs dans les réactions antioxydatives et antiradicalaires.

Mots clés : ecdystéroïde, *Serratula strangulata*, radical libre, érythrocyte, peroxydation lipidique, antioxydant.

[Traduit par la Rédaction]

Introduction

Ecdysteroids were initially recognized as steroidal hormones controlling moulting and metamorphosis in insects, and it was realized recently that these steroids are present in all stages of insect development, regulating many biochemical and physiological processes (Dinan 2001). Since the first isolation of ecdysteroids from plant sources (Nakanishi et al. 1966), phytoecdysteroids have been found in over 100 terrestrial plant families, including ferns, gymnosperms, and angiosperms. Many of them have been reported to possess interesting pharmacological effects, such as control of diabetes (Najmutdinova and Saatov 1999), anticarcinogenesis (Takasaki et al. 1999), and being used in gene replacement therapy to correct inborn errors and used as gene switches (Luers et al. 2000; Albance et al. 2000). However, an antioxidative effect of ecdysteroids has not yet been reported.

It is now commonly recognized that reactive oxygen species are involved in a variety of physiological and pathological processes, including cellular signal transduction, cell proliferation and differentiation, and apoptosis, as well as ischemia–reperfusion injury, inflammation, and many degenerative diseases (Halliwell and Gutteridge 1999; Sen and Packer 1996; Kroemer et al. 1995; Abuja and Albertini 2001). In particular, lipid peroxidation and DNA damage mediated by reactive oxygen species are associated with a variety of chronic health problems, such as cancer, ageing, and atherosclerosis (Marnett 2000; Shigenaga et al. 1994; Bland 1995). Plant- and food-derived antioxidants, such as green-tea polyphenols and resveratrol, have been reported to be beneficial in protecting against these diseases (Ahmad and Mukhtar 1999; Jankun et al. 1997; Jang et al. 1997); hence, antioxidant therapy has become an attractive therapeutic strategy (Rice-Evans et al. 1993). As a part of our ongoing research program on antioxidants (Zhou et al. 2000*a*,*b*; Chen et al. 2001; Liu et al. 2000), we report herein the antioxidative and free radical scavenging effects of four

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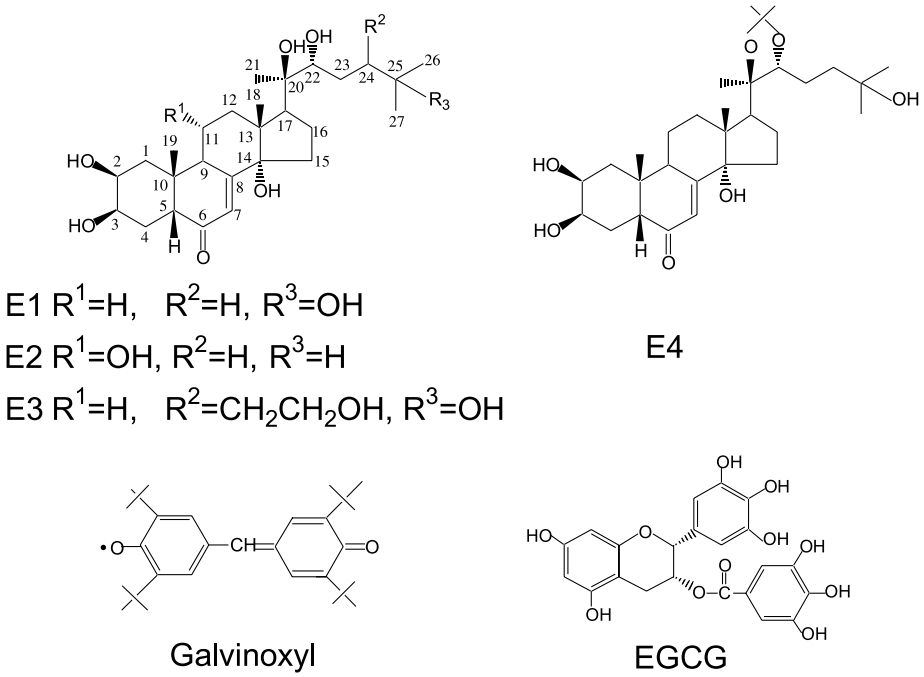
Fig. 1. Molecular structures of the ecdysteroids, galvinoxyl, and epigallocatechin gallate (EGCG).



phytoecdysteroids, 20-hydroxyecdysone (E1), 25-deoxy-11,20-dihydroxyecdysone (E2), 24-(2-hydroxyethyl)-20-hydroxyecdysone (E3), and 20-hydroxyecdysone-20,22-monoacetonide (E4) isolated from *Serratula strangulata* (Dai et al. 2001). Generally, antioxidants are molecules bearing active hydroxyl groups, such as vitamins E and C (Liu 1995) and tea polyphenols (Liu et al. 2000). Therefore, it is interesting to find that these ecdysteroids, which do not have active hydroxyl groups, are also active antioxidants and free-radical scavengers.

Materials and methods

Materials

Ecdysteroids E1–E4 were isolated by repeated column chromatography and preparative thin-layer chromatography of an alcoholic extract of the whole *S. strangulata* plant. Their structures were identified by spectroscopic methods and two-dimensional NMR as 20-hydroxyecdysone (E1), 25-deoxy-11,20-dihydroxyecdysone (E2), 24-(2-hydroxyethyl)-20-hydroxyecdysone (E3), and 20-hydroxyecdysone-20,22-monoacetonide (E4), as reported previously (Fig. 1) (Dai et al. 2001). (–)-Epigallocatechin gallate (EGCG) was isolated from green-tea leaves by extraction with methanol, water, and ethyl acetate, consecutively, and chromatographic separation on a Sephadex LH-20 column, with reference to procedures reported previously (Nonaka et al. 1983). The structures and purity of the compounds were confirmed by [^{1}H]- and [^{13}C]-NMR spectra and HPLC, as reported previously (Fig. 1) (Jia et al. 1998).

Thiobarbituric acid (TBA), butylated hydroxytoluene (BHT), reduced L-glutathione (GSH), and 1,6-diphenyl-1,3,5-hexatriene (DPH) were purchased from Sigma (St. Louis, Mo.); 2,2′-azobis(2-amidinopropane hydrochloride) (AAPH) and galvinoxyl were from Aldrich (Milwaukee, Wisc.); (±)-α-tocopherol was from Merck (Darmstadt, Germany). All other chemicals were of the highest quality available.

Assay for hemolysis of human erythrocytes

Human erythrocytes were separated from heparinized blood that was drawn from a healthy donor. The blood was centrifuged at 1000 × *g* for 10 min to separate the erythrocytes from plasma, and the erythrocytes were washed three times with phosphate-buffered saline (PBS) (pH 7.4). During the last washing, the cells were centrifuged at 1000 × *g* for 10 min to obtain a consistently packed cell preparation. A 5% suspension of human erythrocytes in PBS was preincubated with antioxidants at 37°C for 5 min, to which AAPH was added to initiate hemolysis. The ecdysteroids and α-tocopherol were dissolved in dimethyl sulfoxide (DMSO) before the experiment, and the volume of DMSO solution added to the erythrocyte suspension was less than 0.1% v/v of the reaction mixture. GSH and EGCG were dissolved in PBS and added directly to the reaction mixture. The reaction mixture was gently shaken at 37°C. Aliquots of the reaction mixture was taken out at specific intervals and diluted with 10 volumes of 0.15 M NaCl. The absorbance (A_1) of the supernatant at 540 nm was measured. Similarly, the reaction mixture was treated with distilled water to yield complete hemolysis, and the absorbance (A_2) at 540 nm of the supernatants after centrifugation at 1000 × *g* for 10 min was measured. The percentage hemolysis was calculated from the ratio of the measurements as follows: (A_1/A_2) × 100 (Miki et al. 1987). The lag phase of the oxidative hemolysis was calculated as described by Palozza et al. (1992) by drawing a straight line through the linear portion of the propagation phase until it intercepts the abscissa. Every experiment was repeated three times and the values represent means ± SE of the three experiments.

Preparation of rat liver microsomes

Female Wister rats weighing 250 ± 20 g were starved overnight before cervical dislocation. Liver microsomes were prepared by tissue homogenization with ice-cold STE buffer (0.25 M sucrose – 0.01 M Tris (pH 7.4) with 1 mM EDTA) in a motor-driven glass homogenizer (Greenwald 1985). Microsomal vesicles were isolated by removal of the nuclear faction at 8000 × *g* for 10 min and removal of mitochondrial fraction at 18 000 × *g* for 10 min. The microsomal fraction was sedimented in a Hitachi 55P-72 ultracentrifuge (Hitachi High Technologies, Tokyo, Japan) at 105 000 × *g* for 60 min and washed twice with 0.15 M KCl at 105 000 × *g* for 30 min. The membranes, suspended in 0.1 M potassium phosphate buffer (pH 7.5), were stored in a deep freezer maintained at –20°C. Microsomal protein was determined by the method of Lowry et al. (1951).

Microsomal peroxidation as measured by TBA-reactive substance formation

The formation of TBA-reactive substances (TBARS) was used to monitor lipid peroxidation (Buege and Aust 1978). Rat liver microsomes were incubated at 37°C in 0.1 M potassium phosphate buffer (pH 7.5) and made up to a final protein concentration of 1.0 $mg \cdot mL^{-1}$. The peroxidation was initiated by 50 μM $FeSO_4$ and 200 μM cysteine. After 30 min, 2 mL of TCA–TBA–HCl reagent (15% w/v trichloroacetic acid, 0.37% thiobarbituric acid, and 0.25 N HCl) was added to the reaction mixture, together with 0.02% w/v BHT. This amount of BHT completely prevents the formation of any nonspecific TBARS (Palozza et al. 1992). The solution was heated for 15 min in a boiling water bath. After cooling, the precipitate was removed by centrifugation. The level of TBARS in the supernatant was determined at 532 nm by use of an extinction coefficient of 1.56×10^5 $M^{-1} \cdot cm^{-1}$ (Buege and Aust,1978). Every experiment was repeated three times and the values represent means ± SE of the three experiments.

Microsomal peroxidation as measured by membrane fluidity

The membrane fluidity of rat liver microsomes was measured by fluorescence depolarization using DPH as the lipid probe (Shintzek and Barrenholz 1974). A small volume of DPH solution (2 mM) in tetrahydrofuran was injected with rapid stirring into 1000 volumes of PBS (pH 7.4) at room temperature. The suspension was stirred for 2 h. In a typical experiment, a mixture containing 1.5 mL PBS, 200 μg microsomal protein, 1.5 mL of 2 μM DPH, 100 μM $FeSO_4$, and 750 μM cysteine was incubated at 25°C for 30 min. Fluorescence polarization was measured in a Hitachi 850 spectrofluorimeter (Hitachi High Technologies) equipped with a polarizer, using an excitation wavelength of 362 nm and emission wavelength of 430 nm (Dave et al. 1981). The degree of polarization (*P*) was calculated by the formulas $P = (I_{0,0} - GI_{0,90})/(I_{0,0} + GI_{0,90})$ and $G = I_{90,0}/I_{90,90}$, where $I_{0,0}$ is the fluorescence intensity of the emitted light when the excitation and emission light polarizers are both vertical, $I_{0,90}$ is the fluorescence intensity when the excitation polarizer is vertical and the emission polarizer is horizontal; $I_{90,90}$ is the fluorescence intensity when the the excitation and emission light polarizers are both horizontal; $I_{90,0}$ is the fluorescence intensity when the excitation polarizer is horizontal and the emission polarizer is vertical; and G is a correction factor. A smaller value of *P* demonstrates a greater lipid membrane fluidity.

The microviscosity of membrane fluidity, η, of membranes was calculated by the formula $\eta = 2P/(0.46 - P)$.

Reaction with galvinoxyl radical

The reaction kinetics of galvinoxyl (5 μM) with ecdysteroids (10–100 μM) in ethanol solution were monitored spectrophotometrically at 429 nm (Tsychiya et al. 1985) with a Hitachi model 557 UV spectrometer (Hitachi High Technologies) at 37°C.

Statistical analysis

Values are means ± SE. For most experiments, means were compared using Student's *t* test to evaluate statistical differences.

Results and discussion

Inhibition of AAPH-induced erythrocyte hemolysis by ecdysteroids

Erythrocyte membranes are rich in polyunsaturated fatty acids, which are very susceptible to oxidative stress mediated by free radicals. AAPH is a water-soluble azo compound that could decompose at physiological temperatures to generate alkyl radicals, which can attack membrane lipids from outside of erythrocytes and eventually cause oxidative membrane damage and hemolysis (Miki et al. 1987; Ma et al. 2000*a*,*b*). Since AAPH is water soluble and the rate of free-radical generation from AAPH can be easily controlled and measured, it has been extensively used as a free-radical initiator for biological and related studies (Liu et al. 2000; Zhou et al. 2000*a*,*b*) and the hemolysis induced by AAPH provides a good approach for studying membrane damage induced by free radicals (Miki et al. 1987; Kuang et al. 1994). Human erythrocytes were stable and little hemolysis occurred within 4 h when incubated in PBS under air at 37°C in the absence of AAPH. Addition of AAPH induced fast hemolysis after a short inhibition period, demonstrating lipid peroxidation and the presence of endogenous antioxidants in human erythrocyte membranes, such as α-tocopherol and ubiquinol-10 (Miki et al. 1987), which can trap the initiating and (or) propagating radicals to inhibit hemolysis. It has been proven that the inhibition period is correlated in a concentration-dependent manner with the concentration of AAPH (Ma et al. 2000).

Figures 2 and 3 show the antihemolysis effect of ecdysteroids. It is clearly seen from Fig. 2 that addition of E1 to the human erythrocyte suspension significantly increased the inhibition period of the native erythocytes, and the inhibition period depended on the concentration of E1. Other ecdysteroids also exhibited apparent antihemolysis effects, as exemplified in Fig. 3. The results are listed in Table 1. It is seen from Table 1 that all four of the ecdysteroids are more effective inhibitors of AAPH-induced hemolysis than GSH, a well-known endogenous antioxidant, and E3 is more effective than EGCG, a very active natural polyphenolic antioxidant obtained from green tea (Liu et al. 2000; Zhou et al. 2000*a*,*b*). On the basis of the inhibition period, the

Fig. 2. Inhibition of AAPH-induced hemolysis of human erythrocytes by E1 in a 5% suspension of human erythrocytes in PBS (pH 7.4) under air at 37°C with 51.6 mM AAPH. Values represent means ± SE of three experiments. The initial concentrations of E1 were as follows: a, 0 μM; b, 20 μM; c, 40 μM; and d, 60 μM; e, control with 0 mM AAPH.

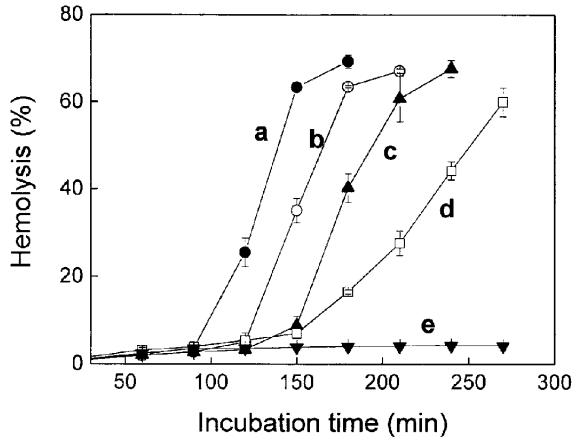
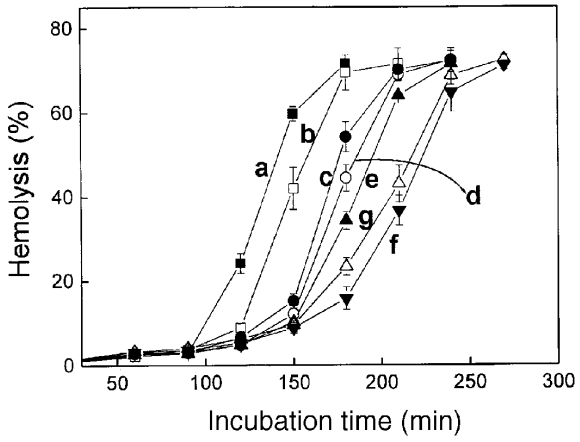


Fig. 3. Inhibition of AAPH-induced hemolysis of human erythrocytes by ecdysteroids, GSH, and EGCG in a 5% suspension of human erythrocytes in PBS (pH 7.4) under air at 37°C. The reaction was initiated with 51.6 mM AAPH and inhibited with 40 μM antioxidants. Values represent means ± SE of three experiments. a, AAPH alone; b, GSH; c, E4; d, E2; e, E1; f, E3; g, EGCG.



inhibitory activity against AAPH-induced erythrocyte hemolysis follows the sequence of E3 > EGCG ≈ E1 > E2 > E4 > GSH.

Inhibition of AAPH-induced erythrocyte hemolysis by the ecdysteroids and exogenous α-tocopherol

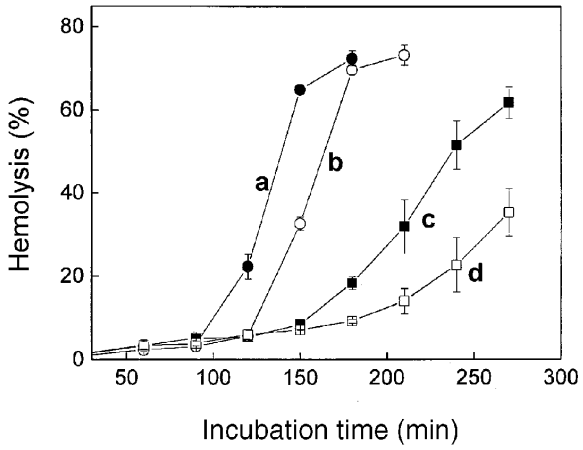
We found recently that green-tea polyphenols exhibited a synergistic antioxidant effect with α-tocopherol in micelles (Zhou et al. 2000*a*,*b*) and human low-density lipoprotein (Liu et al. 2000). The synergistic antioxidative mechanism was proven by demonstrating that green-tea polyphenols reduced α-tocopheroxyl radicals and regenerated α-tocopherol. Therefore, it is desirable to see if the ecdysteroids could also work synergistically with α-tocopherol in their antihemolytic effect. A representative result is shown in Fig. 4. When

Table 1. Effect of antioxidants on the lag phase of AAPH-induced hemolysis of human erythrocytes in vitro.

Antioxidant	Inhibition period (min)
Control	99.3±2.5
E1	162.0±2.0*
E2	144.3±4.0*
E3	171.6±5.0*
E4	137.8±6.5*
GSH	114.7±3.5*
EGCG	162.5±4.5*

Note: The incubation mixture contained 5% human erythrocytes in PBS. Oxidation was initiated by addition of 51.6 mM AAPH. Ecdysteroids were dissolved in DMSO, and GSH and EGCG were added directly to the PBS suspension to a final concentration of 40 μM. Each reaction was carried out after preincubation at 37°C with gentle shaking. Values represent means ± SE of three experiments. *, $p < 0.01$ as compared with the control.

Fig. 4. Inhibition of AAPH-induced hemolysis of human erythrocytes by E1 and α-tocopherol in 5% suspension of human erythrocytes in PBS (pH 7.4) under air at 37°C. The reaction was initiated with 51.6 mM AAPH. Values represent means ± SE. of three experiments. a, AAPH alone; b, 20 μM E1; c, 6 μM α-tocopherol; d, 20 μM E1 and 6 μM α-tocopherol.



20 μM E1 and 6 μM α-tocopherol were added together, the additional inhibition period produced by the two antioxidants was 87 min, approximately the sum of the inhibition periods when the two antioxidants were used individually (25 and 62.5 min, respectively). This implies that E1 and exogenous α-tocopherol work independently in their antihemolytic action, and E1 might not able to reduce α-tocopheroxyl radicals, as in the case of green-tea polyphenols.

Inhibition of hydroxyl radical induced peroxidation of rat liver microsomes by the ecdysteroids

Similar to erythrocyte membranes, microsomes (especially smooth endoplasmic reticulum) are particularly susceptible to oxidative stress because of their high polyunsaturated fatty-acid content (Liebler et al., l997). Iron (Fe^{2+} plus a reducing reagent) is an extensively used system for generating hydroxyl radicals to induce lipid peroxidation, which can be measured by the TBA method (Chattopadhyay

Fig. 5. Concentration-dependent inhibition of thiobarbituric acid reactive substance (TBARS) formation by ecdysteroids during the peroxidation of rat liver microsomes induced by Fe^{2+} (50 μM $FeSO_4$) and cysteine (200 μM) at 37°C. The rat liver microsomes were incubated in 0.1 M potassium phosphate buffer (pH 7.5) at a protein concentration of 1.0 mg·mL^{-1} for 30 min. Values represent means ± SE of three experiments.

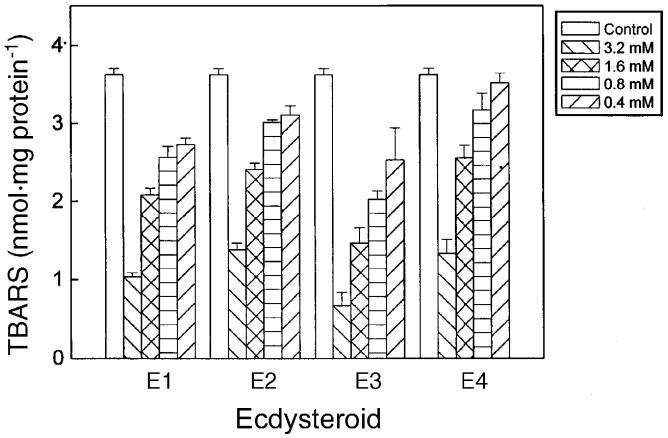
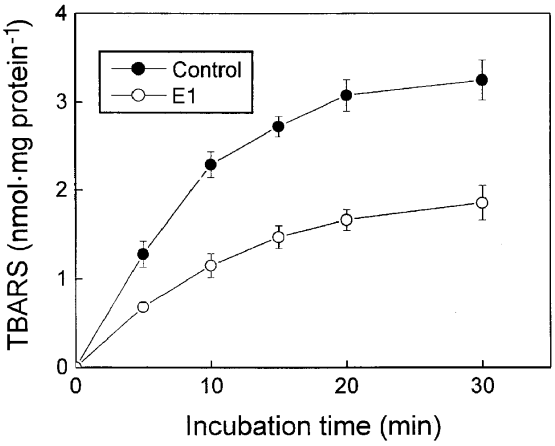


Fig. 6. Time-dependent inhibition of thiobarbituric acid reactive substance (TBARS) formation by E1 during the peroxidation of rat liver microsomes induced by Fe^{2+} (50 μM $FeSO_4$) and cysteine (200 μM) at 37°C. The rat liver microsomes were incubated in 0.1 M potassium phosphate buffer (pH 7.5) at a protein concentration of 1.0 mg·mL^{-1}. Values represent means ± SE of three experiments.



et al. 2000). In the present study, we used Fe^{2+} and cysteine to induce peroxidation of rat liver microsomes to investigate the antioxidant effect of the four ecdysteroids. It was found that the four ecdysteroids preincubated with rat liver microsomes inhibited formation of TBARS in a concentration- and time-dependent manner (Figs. 5 and 6). E3 is the most effective among the four ecdysteroids. The antioxidant activity sequence is E3 > E1 > E2 > E4, similar to that of antihemolysis, mentioned above. However, the concentration of these compounds used in this experiment was 50 times higher than that in the antihemolysis experiment. On the other hand, we also used a NADH – phenazine methosulfate system (Liu and Ng 2000) to generate superoxide radical anions and studied the effect of the four ecdysteroids. It showed that these ecdysteroids have no scavenging effect on superoxide radical anions (data not shown).

Inhibition of hydroxyl radical induced decrease of membrane fluidity of rat liver microsomes by ecdysteroids

It is well known that lipid peroxidation of biomembranes always leads to decreased membrane fluidity and increased membrane microviscosity, since the structure of the biomembrane is disturbed (Dave 1981). DPH is a well-established and efficient lipid probe that can insert into lipid bilayers for monitoring membrane fluidity by fluorescence polarization (Shintzek and Barrenholz 1974). It was found that addition of E1 to the incubation mixture of rat liver microsomes inhibited the reduction of membrane fluidity and the increase of membrane microviscosity caused by Fe^{2+} and cysteine induced lipid peroxidation in a concentration-dependent manner, in the concentration range of 0.2–3.2 mM. (Table 2).

Table 2. Effect of E1 on membrane fluidity of rat liver microsomes for peroxidation induced by Fe^{2+} and cysteine in vitro.

Group	Fluorescence polarization (P)	Microviscosity (η)
Control	0.184±0.021	1.35±0.25
Fe^{2+} and cysteine	0.272±0.020*	2.92±0.53*
Fe^{2+} and cysteine + E1		
3.2 mM E1	0.166±0.017*	1.14±0.18*
0.8 mM E1	0.188±0.035*	1.62±0.56*
0.2 mM E1	0.226±0.024*	1.96±0.42*

Note: The incubation mixture contained 1.5 mL PBS buffer, 200 μg microsomal protein, 2 μM DPH, 100 μM $FeSO_4$, and 750 μM cysteine. Incubation was carried out at 25°C for 30 min. Fluorescence polarization was measured in a Hitachi 850 fluorophotometer. The values represent means ± SE of three experiments. *, probability of $p < 0.01$ as compared with the control.

Free radical scavenging activity of the ecdysteroids as studied by reaction with galvinoxyl

Galvinoxyl is a stable phenoxy radical that exhibits characteristic UV absorption at 429 nm in ethanol solution. This allows easy measurement of the depletion of galvinoxyl radicals in the presence of antioxidants (Tsuchiya et al. 1985; Niki et al. 1985). The kinetic decay of galvinoxyl radicals in homogeneous solution has been used to evaluate the chemical activity of antioxidants of biochemical interest (Gotoh et al. 1992). In the present study, the interaction of ecdysteroids E1–E4 with galvinoxyl was studied in ethanol solution. In the absence of ecdysteroids, the UV spectrum of galvinoxyl was stable under the experimental conditions, and no appreciable decay of its UV spectrum could be observed within 1 h. When the ecdysteroid was added to the ethanol solution of galvinoxyl, the UV spectrum of galvinoxyl diminished gradually with time. A set of representative experimental results is shown in Fig. 7, demonstrating that the ecdysteroids directly scavenged galvinoxyl radicals. The decay was found to be exponential and the pseudo-first-order rate constants (k_{obs}) for the reactions can be obtained from the slope of the linear plot of the logarithm of the concentration of galvinoxyl versus reaction time (Fig. 8). The results are summarized in Table 3. Rate constants for the reaction of gavinoxyl with cysteine and GSH

Fig. 7. UV spectra of galvinoxyl in the presence of E2 in ethanol solution. The spectra were recored every 8 min after mixing 5 μM galvinoxyl and 50 μM E2 under air at 37°C. The arrow at the spectrum peak shows the decrease of the maximum absorbance used for the kinetic determination.

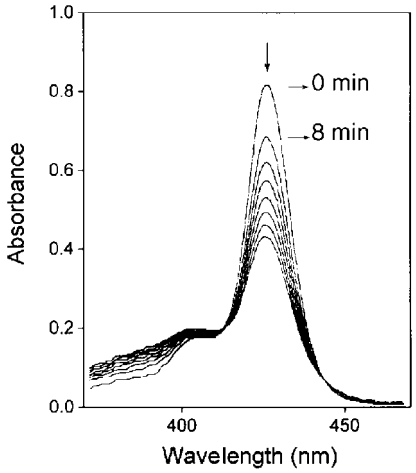


Fig. 8. Pseudo-first-order plot of the decay of galvinoxyl radicals in the reaction with ecdysteroids in ethanol solution under air at 37°C. The initial concentrations of galvinoxyl and the ecdysteroid were 5 and 50 μM, respectively. a, E1; b, E2; c, E3; d, E4; ln C, logarithm of the concentration of galvinoxyl.

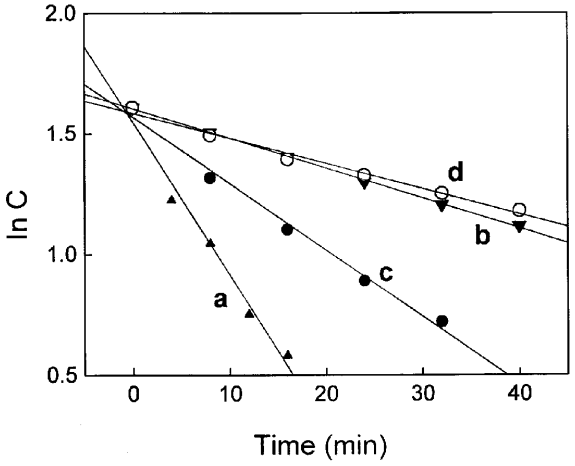
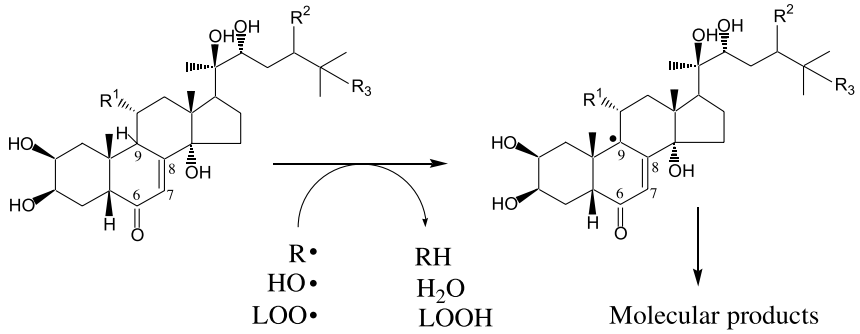


Fig. 9. Proposed antioxidation mechanism of the ecdysteroid.



in homogenous solutions (Tsuchiya et al. 1985) are also listed for comparison.

It is seen from Table 3 that the reaction activity of the ecdysteroids with galvinoxyl follows the sequence of E1 > E3 > E2 > E4, similar to the sequences for their antihemolysis activity and TBARS inhibitory activities, except that E3 is more active than E1 in the latter cases (see above). It is also seen from Table 3 that the galvinoxyl scavenging activity of E1 is comparable to that of cysteine, which is a well known active antioxidant. Compound E4, which possesses the least antihemolysis activity and TBARS inhibitory activity, is also the least active in its galvinoxyl scavenging reaction, and its activity is comparable to that of GSH in both cases.

Table 3. Pseudo-first-order rate constants (k_{obs}) for the interaction of galvinoxyl radicals with ecdysteroids and other antioxidants in ethanol solution.

Antioxidant	k_{obs} ($10^{-3}s^{-1}$)
E1	1.05
E2	0.21
E3	0.46
E4	0.17
Cysteine	1.0*
GSH	0.18*

*Cited from Tsuchiya et al. 1985.

Conclusions

Ecdysteroids E1–E4 obtained from *S. strangulata* are good antioxidants against free radical induced hemolysis of human erythrocytes and against free radical induced lipid peroxidation of rat liver microsomes. They are also effective scavengers for galvinoxyl radicals. However, they are inactive towards superoxide radical anions. These facts suggest that these ecdysteroids can react with the initiating AAPH radical (R·) and hydroxyl radical (HO·), and (or) propagating lipid peroxyl radical (LOO·) to inhibit lipid peroxidation, which in turn inhibits erythrocyte hemolysis and TBARS formation. The most active hydrogen of the ecdysteroid might be H-9, which is an allylic hydrogen. It is well known that allylic hydrogens are very active and easily abstracted by free radicals. In addition, conjugation with the 6-carbonyl group shall further weaken the C–H-9 bond. The bond dissociation energy of the C–H-9 bond of the ecdysteroid can be estimated as 72 kcal·mol^{-1} (Perkins 1994) that is even lower than the bond dissociation energy of the O–H bond of α-tocopherol (76 kcal·mol^{-1} (1 cal = 4.1868 J), Coronel and Colussi 1988). Therefore, the antioxidation mechanism of the ecdysteroids might be rationalized as shown in Fig. 9.

The C-9 radical formed is a tertiary-carbon-centered radical conjugated with a C=C double bond and a C=O double bond that makes the radical very stable. The mechanistic details are worthy of further study.

Acknowledgement

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References

Abuja, P.M., and Albertini, R. 2001. Methods for monitoring oxidative stress, lipid peroxidation and oxidation resistance of lipoproteins. Clin. Chim. Acta, **306**: 1–17.

Ahmad, N., and Mukhtar, H. 1999. Green tea polyphenols and cancer: biologic mechanisms and practical implications. Nutr. Rev. **57**: 78–83.

Albance, C., Reutens, A.T., Bouzahzah, B., Fu, M., D'Amico, M., Link, T., Nicholson, R., Depinho, R., and Pestell, R.G. 2000. Sustained mammary gland-directed, ponasterone A-inducible expression in transgenic mice. FASEB (Fed. Am. Soc. Exp. Biol.) J. **14**: 877–884.

Bland, J.S. 1995. Oxidants and antioxidants in clinical medicine: past, present and future potential. J. Nutr. Envir. Med. **5**: 255–280.

Buege, J.A., and Aust, S.D. 1978. Microsomal lipid peroxidation. Methods Enzymol. **52**: 302–310.

Chattopadhyay, A., Choudhury, T.D., Bandyopadhyay, D., and Datta, A.G. 2000. Protective effect of erythroprotein on the oxidative damage of erythrocyte membrane by hydroxyl radical. Biochem. Pharmacol. **59**: 419–425.

Chen, Z.H., Zhou, B., Yang, L., Wu, L.M., and Liu, Z.L. 2001. Antioxidnat activity of green tea polyphenols against lipid peroxidation initiated by lipid-soluble radicals in micelles. J. Chem. Soc. Perkin Trans. I, **2**: 1835–1839.

Coronel, M.E.J., and Colussi, A.1988. ESR equilibrium measurements of the O–H bond energy in α-tocopherol. J. Intern. J. Chem. Kinetics, **20**: 749–752.

Dai, J.Q., Zhu, Q.X., Zhao, C.Y., Yang, L., Liu, Z.L., and Li, Y. 2001. Glyceroglycolipids from *Serratula strangulata*. Phytochemistry, **58**: 1305–1309.

Dave, J.R., Knazek, R.A., and Liu, S.C. 1981. Prolactin modifies the fluidity of rat liver membranes. Biochem. Biophys. Res. Commun. **100**: 45–51.

Dinan, L. 2001. Phytoecdysteroids: biological aspects. Phytochemistry, **57**: 325–339.

Gotoh, N., Shimizu, K., Komuro, E., Tsuchiya, J., Noguchi, N., and Niki, E. 1992. Antioxidant activities of probucol against lipid peroxidations. Biochim. Biophys. Acta, **1128**: 147–154.

Greenwald, R.A. 1985. CRC handbook of methods for oxygen radical research. CRC Press. Inc. Boca Raton, Fl. pp. 82–83.

Halliwell, B., and Gutteridge, J.M.C. 1999. Free radicals in biology and medicine. 3rd ed. Oxford Science Publications. Clarendon Press, Oxford.

Jang, M., Cai, L., Udeani, G.O., Slowing, K.V., Thomas, C.F., Beecher, C.W.W., Fong, H.H.S., Farnsworth, N.R., Kinghorn, A.D., Mehta, R.G., Moon, R.C., and Pezzuto, J.M. 1997. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. Science (Washington, D.C.), **275**: 218–219.

Jankun, J., Selman, S.H., Swiercz, R., and Skrzypczak-Jankun, E. 1997. Why drinking green tea could prevent cancer. Nature (London), **387**: 561.

Jia, Z.S., Zhou, B., Yang, L., Wu, L.M., and Liu, Z.L. 1998. 2D NMR study on tea polyphenols. Chin. J. Magn. Reson. **15**: 23–30.

Kroemer, G., Pett, P., Zamzami, N., Vayssiere, J.L., and Mignotte, B. 1995. The biochemistry of programmed cell death. FASEB (Fed. Am. Soc. Exp. Biol.) J. **9**: 1277–1287.

Kuang, Z.H., Wang, P.F., Zheng, R.L., Liu, Z.L., and Liu, Y.C. 1994. Making vitamin C lipo-soluble enhances its protective effect against radical induced hemolysis of erythrocytes. Chem. Phys. Lipids, **71**: 95–98.

Liebler, D.C., Stratton, S.P., and Kaysen, K.L. 1997. Antioxidant actions of β–carotene in liposomal and microsomal membranes: role of carotenoid-membrane incorporation and α-tocopherol. Arch. Biochem. Biophys. **338**: 244–250.

Liu, F., and Ng, T.B. 2000. Antioxidative and free radical scavenging activities of selected medicinal herbs. Life. Sci. **66**: 725–735.

Liu, Z.L. 1995. Antioxidant activity of vitamin E and vitamin C derivatives in membrane mimetic systems. *In* Bioradicals detected by ESR spectroscopy. *Edited by* H. Ohya-Nishiguchi and L. Packer. Birkhauser Verlag, Basel, pp. 259–275.

Liu, Z.Q., Ma, L.P., Zhou, B., Yang, L., and Liu, Z.L. 2000. Antioxidative effects of green tea polyphenols on free radical initiated and photosensitized peroxidation of human low density lipoprotein. Chem. Phys. Lipids, **106**: 53–63.

Lowry, O.H., Rosebrough, M.J., Farr, A., and Randall, R.J. 1951. Protein measurement with folin phenol regent. J. Biol. Chem. **193**: 265–275.

Luers, G.S., Jess, H., and Franz, T. 2000. Reporter-linked monitoring of transgene expression in living cells using the ecdysone-inducible promoter system. Eur. J. Cell. Biol. **79**: 653–657.

Ma, L.P., Liu, Z.Q., Zhou, B., Yang, L., and Liu, Z.L. 2000. Inhibition of free radical induced oxidative hemolysis of red blood cells by green tea polyphenols. Chin. Sci. Bull. **45**: 2052–2056.

Marnett, L. 2000. Oxyradicals and DNA damage. Carcinogenesis, **21**: 361–370.

Miki, M., Tamai, H., Mino, M., Yamamoto, Y., and Niki, E. 1987. Free-radical chain oxidation of rat red blood cells by molecular oxygen and its inhibition by α-tocopherol. Arch. Biochem. Biophys. **258**: 373–380.

Najmutdinova, D.K., and Saatov, Z. 1999. Lung local defense in experimental diabetes mellitus and the effect of 11, 20-dihydroxyecdysone in combination with maninil. Arch. Insect Biochem. Physiol. **41**: 144–147.

Nakanishi. K., Koreeda. M., Sasaki, L., Chang. M. L., and Hsu. H. Y. 1966. Insect hormones. I. The structure of ponasterone A, an insect molting hormone from the leaves of *Podocarpus nakaii* H. J. Chem. Soc. Chem. Commun. pp. 915–917.

Niki, E., Kawakami, A., Saito, M., Yamamoto, Y., Tsuchiya, J., and Kamiya, Y. 1985. Effect of phytyl side chain of vitamin E on its antioxidant activity. J. Biol. Chem. **260**: 2191–2196.

Nonaka, G., Kawakami, O., and Nishioka, I. 1983. Tannins and related compounds. XV. A new class of dimeric flanan-3-olgallates, theasineneins A and B, and proanthocyanidin gallates from green tea leaf. Chem. Pharm. Bull. **31**: 3906–3910.

Palozza, P., Moualla, S., and Krinsky, N. 1992. Effects of β-carotene and α-tocopherol on radical-initiated peroxidation of microsomes. Free. Rad. Biol. Med. **13**: 127–136.

Perkins, M. J. 1994. Free radical chemistry. Ellis Horwood, New York. p.37.

Rice-Evans, C.A., and Diplock, A.T. 1993. Current status of antioxidant therapy. Free Rad. Biol. Med. **15**: 77.

Sen, C.K., and Packer, L. 1996. Antioxidant and redox regulation of gene transcription. FASEB (Fed. Am. Soc. Exp. Biol.) J. **10**: 709–720.

Shigenaga, M.K., Hagen, T.M., and Ames, B.N. 1994. Oxidative damage and mitochondrial decay in aging. Proc. Natl. Acad. Sci. U.S.A. **91**: 10 771 – 10 778.

Shintzek, M., and Barrenholz, Y. 1974. Dynamics of the hydrocarbon layer in liposomes of lecithin and sphingomyelin containing dicetylphosphate. J. Biol. Chem. **249**: 2652–2657.

Takasaki, M., Tokuda, H., Nishino, H., and Konoshima, T. 1999. Cancer chemopreventative agents (antitumor-promoters) from *Ajuga decumbens*. J. Nat. Prod. **62**: 972–975.

Tsuchiya, J., Yamada, T., Nike, E., and Kamiya, Y. 1985. Interaction of galvinoxyl radical with ascorbic acid, cysteine, and glutathione in homogeneous solutions and in aqueous dispersions. Bull. Chem. Soc. Jpn. **58**: 326–330.

Zhou, B., Chen, Z., Jia, Z., Jia, Y., Zeng, L., Wu, L., Yang, L., and Liu, Z.L. 2000*a*. Kinetic EPR studies on bio-antioxidants. Appl. Magn. Reson. **18**: 397–406.

Zhou, B., Jia, Z.S., Chen, Z.H., Yang, L., Wu, L.M., and Liu, Z.L. 2000*b*. Synergistic antioxidant effect of green tea polyphenols with α-tocopherol on free radical initiated peroxidation of linoleic acid in micelles. J. Chem. Soc. Perkin Trans. I, **2**: 785–791.