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Antioxidant and Antiviral Activities of *Euphorbia thymifolia* L.

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Key Words
Euphorbia thymifolia · Antioxidant activity · Anti-HSV-2 activity

Abstract
The antioxidant and antiviral activities of *Euphorbia thymifolia* L. (Euphorbiaceae) were investigated in this study. The results showed that all of the fractions (MeOH, $CHCl_3$, EtOAc, n-butanol and water) and pure compounds (3-*O*-galloyl-4,6-*(S)*-HHDP-*D*-glucose, rugosin B and 1,3,4,6-tetra-*O*-galloyl-K-β-*D*-glucose)tested possessed antioxidant activities, with the exception of the organic aqueous fraction in the anti-lipid and anti-superoxide formation assays. The range of IC_{50} of anti-lipid formation, anti-superoxide formation and free radical scavenging assays for all fractions and pure compounds were 2.81–7.63, 0.03–2.18 and 0.013–2.878 mg/ml, respectively. Electron spin resonance studies showed that water extract and pure compounds of *E. thymifolia* exhibited superoxide radical and hydroxyl radical scavenging activities. Besides antioxidant activities, 3-*O*-galloyl-4,6-*(S)*-HHDP-*D*-glucose and EtOAc fraction also showed anti-HSV-2 activity. Thus, *E. thymifolia* was concluded to possess antioxidant and anti-HSV-2 activities.

Introduction

Euphorbia thymifolia (Euphorbiaceae), also known as *Chamaesyce thymifolia* L., is commonly used as a herbal medicine in Taiwan. It is believed to possess diuretic, laxative, detumescent, anti-diarrheic, anti-malarial, anti-rash, anti-dysentery, anti-carbuncle, detoxification and anti-hemorrhoidal activities.

According to previous reports, extract of *E. thymifolia* prepared with 1.5% HCl could inhibit the growth of gram-positive *(Bacillus subtilis)* and gram-negative *(Escherichia coli)* bacteria [7]. Ether acetate and chloroform extracts of this plant were also reported to exhibit activity against the growth of *E. coli* and *Shigella flexneri* [8]. Studies also showed that ether acetate extract of *E. thymifolia* possesses antifungal activity [12, 27]. In 1982, human subjects with contact dermatitis treated with extract of this plant exhibited positive responses [3]. *E. thymifolia* has also shown beneficial effects when used in the treatment of diarrhea and dysentery [9].

Previous studies isolated only 16 tannins from *E. thymifolia* [13], of which 3-*O*-galloyl-4,6-*(S)*-HHDP-*D*-glucose, rugosin B and 1,3,4,6-tetra-*O*-galloyl-β-*D*-glucose were used in this study. Rugosin B was reported to reduce the minimum inhibitory concentration of β-lactams in methicillin-resistant *Staphylococcus aureus* [30]. 1,3,4,6-

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tetra-*O*-galloyl-*β*-*D*-glucose was shown to inhibit chitin synthase II activity [6]. No biological activity of 3-*O*-galloyl-4,6-*(S)*-HHDP-*D*-glucose has been reported.

Serious attention is now paid to the cytotoxicity of active oxygen free radicals which are believed cause various pathological conditions. Free radicals produced from unsaturated fatty acids are well known to cause histotoxicity and promote the formation of additional free radicals in a chain reaction type manner. It is thought that, if the in vivo activity of enzyme or free radical scavengers is not high enough to inhibit free radicals, various diseases such as arteriosclerosis, liver disease, diabetes, inflammation, renal failure or accelerated aging may result [22].

Oxygen radicals, which are generated by the host's immune system during invasion by bacteria and microorganisms, have been shown to possess antimicrobial effect and to enhance the immune system in eliminating pathogens [2]. However, oxygen radicals are not necessarily beneficial to the host during viral infection, as they can cause pathological effects [1]. A previous study showed that the amount of free radicals in alveolar phagocytic cells of influenza-infected mice was significantly increased when compared with noninfected control mice [23]. The survival rate of the influenza-infected mice was improved by injection of pyran copolymer-conjugated SOD, which served as a free radical scavenger. This finding suggests that compounds which exhibit antioxidant activity might reduce viral infection.

Since many diseases are related to excess free radicals and viral infection is known to induce the production of oxygen radicals, the present study was conducted to elucidate the antioxidant and antiviral activities of *E. thymifolia*.

Materials and Methods

Chemicals

L(+)-ascorbic acid (AA), iron(II) chloride anhydrous, thiobarbituric acid (TBA), sodium dodecyl sulfate (SDS), cytochrome C, and xanthine were purchased from Sigma Chemical Co. (USA). 5,5-Dimethyl-1-pyrroline N-oxide (DMPO) and xanthine oxidase (XOD) were purchased from Labotec Co., Ltd. (Tokyo, Japan) and Boehringer Mannheim, respectively.

Plant Material

The dried matured herb of *E. thymifolia* was collected from a local market in Taiwan. It was identified by comparative anatomical studies with equivalent specimens preserved in the Kaohsiung Medical University Herbarium, and further authenticated by C.C. Lin of the College of Pharmacy of Kaohsiung Medical University.

Preparation of Extract

Fifty grams of dried herbal *E. thymifolia* was cut into pieces and extracted with warm H_2O (1 liter × 3). The decoction was filtered, concentrated and lyophilized to yield 14.45 g of dried aqueous extract.

Another 238 g of dried *E. thymifolia* was cut into pieces and then extracted with 1 liter of warm MeOH. The decoction was concentrated under reduced pressure and lyophilized to yield 26.53 g of MeOH extract. 16.49 g of MeOH extract was resuspended in 500 ml H_2O and then extracted successively with $CHCl_3$, EtOAc and n-butanol. The final yield of $CHCl_3$, EtOAc and n-butanol extracts was 3.47, 2.16 and 3.90 g, respectively. Residual solvent was collected and concentrated under reduced pressure. After lyophilization, 2.68 g of organic aqueous extract was obtained.

Besides the extracts described above, 3-*O*-galloyl-4,6-*(S)*-HHDP-*D*-glucose, 1,3,4,6-tetra-*O*-galloyl-β-*D*-glucose and rugosin B, purified from *E. thymifolia* [13], were tested. The structures of 3-*O*-galloyl-4,6-*(S)*-HHDP-*D*-glucose, rugosin B and 1,3,4,6-tetra-*O*-galloyl-*β*-*D*-glucose are shown in figure 1.

Test Animals

Male Wistar Albion rats, which were 4–6 weeks old, were purchased from the National Laboratory Animal Breeding and Research Center of the National Science Council of Taiwan. The animals were housed in an air-conditioned room with environment maintained at a temperature of 22 ± 3 °C, humidity of 55 ± 5% and a 12 h dark-light cycle. The rats were fed with a standard laboratory diet and had access to tap water ad libitum.

Preparation of Liver Homogenate

Liver homogenate was prepared according to a previously described method with minor modification [18]. Briefly, rats that weighed 180–230 g were sacrificed. The liver was quickly removed and cut into pieces. The liver sample was then homogenized in 150 m*M* Tris-HCl (pH 7.2) with a disperser (Ultra-Turrax T25, IKA-Labortechnik) at 20,500 *g* for 3 min to give a 20% (w/v) liver homogenate. The liver homogenate was further centrifuged at 500 *g* for another 10 min. Supernatant of the liver homogenate was collected and the amount of protein was determined using the *DC*™ Protein Assay kit (Bio-Rad) according to the protocols recommended by the manufacturer.

Viruses and Cells

An African green monkey kidney cell line (Vero) was obtained from the hospital of Kaohsiung Medical University and used for antiviral assay. Vero was propagated in DMEM (Gibco BRL), supplemented with 5% fetal calf serum (FCS). Herpes simplex virus type-2 (HSV-2) strain 196 was kindly provided by Dr. Lien-Chai Chiang (Department of Microbiology, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan). Virus stocks were prepared and quantified on Vero cells and stored in small aliquots at −80 °C.

Titration of Virus

Viral titer was determined by plaque assay as described by Burleson et al. [4]. Vero cells were seeded in 24-well culture plates at a density of 10^5 cells/well and incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 48 h until they reached at least 95% confluency. Serial dilution of virus stock was prepared, and the cell monolayer was infected with the dilution of virus. After 1 h of

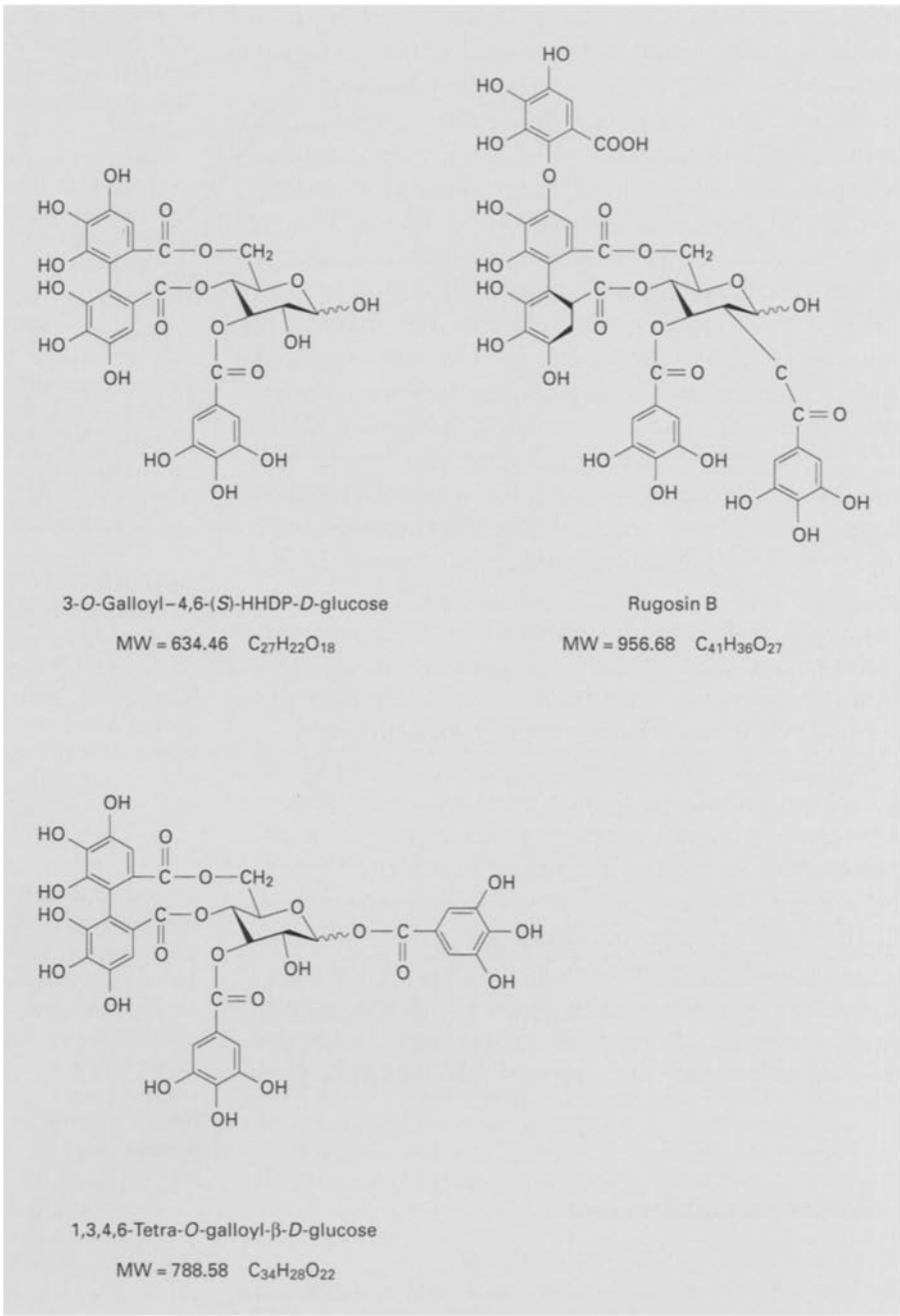

Fig. 1. Structure of three pure compounds of *E. thymifolia*.

inoculation, the medium was aspirated and replaced with overlay medium containing 1% methylcellulose. The infected cell monolayer was incubated for another 48 h. The overlay medium was removed and the cell monolayer was fixed with 10% formalin in PBS for 1 h. After fixation, 1% crystal violet was used to stain the cell monolayer. The plaque number per well was recorded and the virus titer in plaque-forming units (PFU) was calculated.

Anti-Lipid Peroxidation Activity

The anti-lipid peroxidation activity of *E. thymifolia* was evaluated according to the method of Ohkawa et al. [25] and Schinella et al. [28]. The reaction mixture, which comprised 0.25 ml supernatant of liver homogenate (1 mg protein), 0.10 ml Tris-HCl buffer (pH 7.2), 0.05 ml of test extract and 0.10 ml of $FeCl_2$-ascorbic acid to induce nonenzymatic lipid peroxidation for the production of malondialdehyde (MDA), was incubated at 37 °C for 1 h in a capped tube. 0.5 ml of 0.1 *M* HCl, 0.2 ml of 9.8% SDS, 0.9 ml of distilled water and 2.0 ml of 0.6% TBA were then added to each tube. The tube was

vigorously shaken before it was placed in a boiling water bath (100°C) for another 30 min. After cooling, 5 ml n-BuOH was added into the tube and then centrifuged at 3,000 rpm for 25 min to remove flocculent precipitate. Lipid peroxide concentration was determined by MDA-TBA adduct (completion of malondialdehyde with thiobarbituric acid) at 532 nm using a Hitachi U-2000 spectrophotometer. To evaluate the anti-lipid peroxidation activity of *E. thymifolia*, its fractions and pure compounds were incubated together with rat liver homogenate and $FeCl_2$-ascorbic acid.

Anti-Superoxide Radical Formation

Anti-superoxide radical formation activity of *E. thymifolia* was evaluated by spectrophotometric measurement of the formation of uric acid from xanthine/xanthine oxidase system [5, 15]. The samples were first dissolved with DMSO, and then diluted to the desired concentrations with PBS. 50 µl of sample solution, 400 µl of xanthine/PBS and 530 µl of H_2O were added into the tube. The tube was then vigorously mixed, and 20 µl of 1-unit xanthine oxidase solution (1 unit of xanthine oxidase in 1 ml of PBS) was then added. After vigorously mixing, the solution was screened for 1 min at 295 nm. The IC_{50} of each sample was calculated from the regression line.

Free Radical Scavenging Activity

Free radical scavenging activity was assayed spectrophotometrically by the cytochrome C reduction method as described by McCord and Fridovich [19] and Yu et al., [35]. When xanthine oxidase converts xanthine to uric acid, the superoxide anion produced would reduce ferricytochrome C to ferrocytochrome C. Since ferrocytochrome C shows maximum absorption at 550 nm, the amount of superoxide anion can be evaluated indirectly by spectrophotometric measurement of ferrocytochrome C. Therefore, the superoxide anion scavenging activity of *E. thymifolia* can be evaluated using the cytochrome C reduction method. Briefly, samples were dissolved in DMSO, and diluted to various concentrations with PBS. Fifty microliters of sample solution, 400 µl of working solution (xanthine/cytochrome C), 530 µl of distilled H_2O and 20 µl of 1-unit xanthine oxidase solution (1 unit of xanthine oxidase in 1 ml of PBS) were mixed vigorously and then screened for 1 min at 550 nm. The IC_{50} of each sample was calculated from the regression line [14].

Superoxide Anion and Hydroxyl Radical Scavenging Activity

Superoxide radical was generated from the hypoxanthine-xanthine oxidase reaction system and trapped by DMPO. The product, spin adduct (DMPO-OOH), was analyzed using an electron spin resonance (ESR) spectrometer [10, 16, 20]. Solutions of 2.0 m*M* HPX/PBS (A), 5.5 m*M* DETAPAC (B), various concentrations of test extracts or SOD (C) and 0.4 unit/ml XOD/PBS (D) were prepared before use. Solution D was stored in an ice bath to prevent any inactivation of enzyme. 50 µl of A, 35 µl of B, 50 µl of C and 15 µl of DMPO were transferred into a test tube. Fifty microliters of D was then added to the mixed solution to produce DMPO-OOH.

During hydroxyl radical scavenging studies, 1 m*M* ferrous sulfate (A), 5.5 m*M* DETAPAC (B), various concentrations of test extracts or ascorbic acid (C), and 1 m*M* hydrogen peroxide (D) were prepared just before use. 37.5 µl of A, 37.5 µl of B, 50 µl of C and 20 µl of DMPO were pipetted into a test tube. Seventy-five microliters of D was then added to the mixed solution to generate spin adduct (DMPO-OH). DMPO-OH was analyzed using an ESR spectrometer.

The reaction mixture prepared to generate either DMPO-OOH or DMPO-OH was stirred and transferred into a quartz analyzing cell, and then placed into the cavity of an ESR spectrometer (Jeol-JES-FR80, Jeol Ltd., Tokyo, Japan). Forty seconds after the addition of XOD and hydrogen peroxide, the reaction mixture was analyzed and the relative intensity of the spin adduct (DMPO-OOH or DMPO-OH) signal was measured as the ratio to the intensity of Mn^{2+} signal. ESR spectra were recorded at 37°C with a field set at 335.4 ± 5.0 mT, modulation frequency 100 kHz, modulation amplitude 0.79 × 0.1 mT, response time 0.1 s, sweep time 2 min, microwave power 8.0 mW (9.416 GHz), and receiver gain 2 × 100 or 1 × 100 for superoxide radical trapping or hydroxyl radical trapping, respectively.

Antiviral Assay

XTT Assay. The antiviral activity of fractions and pure compounds from *E. thymifolia* was assayed using XTT (sodium 3′-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid) methods as described by Weislow et al. [33]. Briefly, 10^4 cells/well were seeded into 96-well culture plates. After 4 h of incubation, the cell monolayer was infected with HSV-2 at multiplicity of infection (MOI) = 0.5. Various concentrations of fractions and pure compounds were then added. The plate was incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 72 h. The medium was then aspirated and XTT reagent (Sigma) was added. The trays were reincubated for an additional 2 h to allow the production of formazan. Optical densities were measured with an EIA reader (Lab Systems) at a test wavelength of 492 nm and a reference wavelength of 690 nm. The antiviral activity of each fraction or pure compound from *E. thymifolia* was determined by the following formula [26]:

$$\text{Antiviralactivity, \%} = \frac{(OD_T)_{HSV} - (ODc)_{HSV}}{(OD_C)_{mock} - (ODc)_{HSV}} \times 100\%$$

where $(OD_T)_{HSV}$ is the optical density measured with a given concentration of the fractions or pure compounds from *E. thymifolia* for HSV-infected cells; $(OD_C)_{HSV}$ is the optical density measured for the control untreated HSV-infected cells; $(OD_C)_{mock}$ is the optical density measured for the control untreated mock-infected cells. The minimum concentration of each fraction or pure compound from *E. thymifolia*, which was required to inhibit 50% of virus growth (IC_{50}) was also calculated.

Plaque Reduction Assay. A plaque reduction assay was performed according to previously described procedures [11] with minor modifications. Vero cells were seeded into 24-well culture plates (Falcon) at a density of 10^5 cells/well and incubated at 37°C with 5% CO_2 until reaching at least 95% confluency. The cell monolayer was then infected with 100 PFU HSV-2 in the absence or presence of fractions and pure compounds from *E. thymifolia* and further incubated for 1 h at 37°C with 5% CO_2. After 1 h of adsorption, the cell monolayer was overlaid with overlay medium. The overlay medium was removed 2 days later, and the infected cell monolayer was fixed and stained with 10% formalin and 1% crystal violet, respectively. The antiviral activity of fractions and pure compounds from *E. thymifolia* was determined by the following formula:

$$\text{Percent of inhibition} = \left[1 - \frac{(\text{number of plaque})_{tested}}{(\text{number of plaque})_{control}}\right] \times 100\%$$

The minimal concentration of fractions and pure compounds from *E. thymifolia* required to reduce the plaque number by 50% (IC_{50}) was calculated by regression analysis of the dose-response curves generated from the data [17].

Table 1. Anti-lipid peroxidation, anti-superoxide radical formation, and free radical scavenging activities of *E. thymifolia* in vitro

Fractions and compounds	IC_{50}, mg/ml		
	anti-lipid peroxidation activity	anti-superoxide radical formation activity	free radical scavenging activity
MeOH fraction	7.63±0.58	0.76±0.04	0.054±0.008
$CHCl_3$ fraction	4.23±0.21	1.31±0.17	0.069±0.010
EtOAc fraction	4.27±0.43	0.71±0.12	0.019±0.002
n-Butanol fraction	2.81±0.43	2.18±0.40	0.013±0.001
Organic aqueous fraction	>20.00	>20.00	0.386±0.066
Water extract	5.69±0.32	0.03±0.004	0.410±0.021
3-*O*-Galloyl-4, 6-(*S*)-HHDP-*D*-glucose	3.63±0.30	1.48±0.30	0.049±0.006
Rugosin B	3.53±0.46	0.34±0.03	0.022±0.001
1,3,4,6-Tetra-*O*-galloyl-β-*D*-glucose	4.02±0.56	0.47±0.05	2.878±0.432
Allopurinol	ND	0.11±0.01	ND
Trolox	0.88±0.07	ND	ND

Each value represents the mean ± SD of three independent experiments. ND = Not done.

Cytotoxicity Assay

The effect of fractions or pure compounds from *E. thymifolia* against Vero cell viability was measured using the XTT method [29]. The cytotoxic concentration of each extract toward Vero cell lines was calculated by the following formula:

$$\text{Percent of survival cell} = \frac{OD_T}{OD_C} \times 100\%$$

where OD_T and OD_C indicate the absorbances of the test compounds and the solvent control, respectively. The 50% cytotoxic concentration (CC_{50}), which was expressed as the concentration that achieved 50% cytotoxicity against Vero cell lines, was calculated from the regression line.

Evaluation of Selectivity Index

With the IC_{50} and CC_{50} data, the selectivity index for each fraction and pure compound from *E. thymifolia* against HSV-2 was evaluated as follows:

$$\text{Selectivity Index} = \frac{CC_{50}}{IC_{50}}$$

Table 2. ESR signal activity of Mn^{2+} and superoxide radical in various concentrations of SOD

SOD units/ml	ESR signal peak height		Averaged relative peak height[a]
	Mn^{2+}	radical	
0.000	101.20	306.20	3.03
1.897	100.20	219.80	2.19
4.743	100.20	131.40	1.31
9.486	98.80	85.20	0.86
14.228	99.80	65.20	0.65
18.971	100.00	50.00	0.53

Calibration curve: y = 0.253x − 0.024 (r = 0.998), where y = $(I_0/I - 1)$; x = concentration of SOD (units/ml); I_0 indicates the relative peak height when the concentration of SOD was zero; I indicates the relative peak height in various concentrations of SOD.

[a] Averaged relative peak height was determined as the ratio of the peak height of radical/Mn^{2+}.

Results

Anti-Lipid Peroxidation, Anti-Superoxide Formation, Superoxide Anion and Hydroxyl Radical Scavenging Activities of E. thymifolia

The anti-lipid peroxidation activity of *E. thymifolia* is shown in table 1. The IC_{50} for fractions and pure compounds of *E. thymifolia* ranged from 2.81 to 7.63 mg/ml. All test compounds showed anti-lipid peroxidation activity with the exception of the organic aqueous fraction, for which the IC_{50} value was higher than 20.00 mg/ml.

Besides anti-lipid peroxidation, *E. thymifolia* was also being investigated for its anti-superoxide radical formation activity. As shown in table 1, both fractions and pure compounds of *E. thymifolia* exhibited anti-superoxide formation activity with the exception of organic aqueous fraction, which had an IC_{50} higher than 20.00 mg/ml. It is interesting to note that water extract showed a lower IC_{50}

Table 3. SOD-like (superoxide radical scavenging) activity of *E. thymifolia*

Sample	Concentration g/ml	ESR signal peak height		SOD activity units/ml	SOD-like activity[a] units/g	IC_{50}[b] mg/ml
		Mn^{2+}	radical			
Water extract	2.51×10^{-2}	98.20	88.20	9.28	3.70×10^{2}	10.44
3-*O*-Galloyl-4, 6-(*S*)-HHDP-*D*-glucose	3.33×10^{-3}	98.40	80.20	10.64	3.20×10^{3}	1.21
Rugosin B	4.90×10^{-3}	99.00	85.60	9.80	2.00×10^{3}	1.93
1,3,4,6-Tetra-*O*-galloyl-*β*-D-glucose	1.25×10^{-2}	99.60	86.40	9.75	7.80×10^{2}	4.95

[a] Calibration curve: $y = 0.253x - 0.024$, where $y = (I_0/I - 1)$; $I_0 = 3.03$ (from table 4); I indicates the average relative peak height, which was the ratio of the peak height of radical /Mn^{2+} in various concentrations of test samples; x = concentration of SOD (unit/ml); SOD-like activity = SOD (units/ml)/the concentration of test samples.

[b] IC_{50} indicates the amount of test samples that caused 50% inhibition of the ESR signal intensity of DMPO-OOH.

value than allopurinol in anti-superoxide formation assay.

Since *E. thymifolia* was shown to inhibit the peroxidation of lipid and the formation of superoxide radical, it was further evaluated for its free radical scavenging activity. Interestingly, all test compounds showed free radical scavenger activity (table 1). The IC_{50} for fractions and pure compounds of *E. thymifolia* ranged from 0.013 to 2.878 mg/ml. Among them, 1,3,4,6-tetra-*O*-galloyl-*β*-*D*-glucose had the highest IC_{50} value (2.878 mg/ml). Its IC_{50} was at least 50-fold higher than other tested compounds. Thus, 1, 3,4,6-tetra-*O*-galloyl-*β*-*D*-glucose was concluded to exhibit weak free radical scavenging activity.

The ESR spin-trapping technique is a powerful tool for investigating superoxide and hydroxyl radical scavenging potency. When DMPO was added to a solution of the HPX-XOD reaction system, the spin adduct DMPO-OOH was formed. The signal intensity of DMPO-OOH was decreased with the addition of SOD in a dose-dependent manner (table 2). This is because SOD can inhibit the reaction between $O_2^{\cdot}$ and DMPO. The linear calibration curve ($y = 0.253x - 0.024$) obtained using a standard SOD solution (0.000–18.971 units/ml in concentration) was determined with peak intensities of the internal standard signal in Mn^{2+}. When fractions and pure compounds of *E. thymifolia* were added to the HPX-XOD reaction system, the signal intensity of DMPO-OOH was decreased. The superoxide radical scavenging activity of *E. thymifolia* was calculated by comparison of the average relative peak height between *E. thymifolia* and standard SOD. The IC_{50} values of SOD-like activity (the amount of SOD that causes 50% inhibition of the ESR signal intensity of DMPO-OOH) for water extract, 3-*O*-galloyl-4,6-*(S)*-HHDP-*D*-glucose, rugosin B and 1,3,4,6-tetra-*O*-galloyl-*β*-*D*-glucose were 10.44, 1.21 1.93 and 4.95 mg/ml, respectively (table 3).

Table 4. ESR signal activity of Mn^{2+} and hydroxyl radical in various concentrations of ascorbic acid

Ascorbic acid m*M*	ESR signal peak height		Averaged relative peak height[a]
	Mn^{2+}	radical	
0.000	53.40	663.40	12.42
0.200	53.40	626.00	11.72
0.400	52.20	517.40	9.91
0.600	52.80	367.60	6.96
0.800	53.20	240.80	4.53
1.000	53.20	133.60	2.51

Calibration curve: $y = 0.060x^3 - 0.363x^2 + 0.794x - 0.013$, where y = concentration of ascorbic acid (m*M*), $x = (I_0/I - 1)$; I_0 indicates the relative peak height when the concentration of ascorbic acid was zero; I indicates the relative peak height in various concentrations of ascorbic acid.

[a] Average relative peak height was determined as the ratio of the peak height of radical/Mn^{2+}.

During the hydroxyl radical scavenging assay, the spin adduct DMPO-OH was used for evaluation. DMPO-OH was formed by the addition of DMPO into the solution of the ferrous sulfate-hydrogen peroxide reaction system. Ascorbic acid, a scavenger of hydroxyl radicals, was added and the signal decayed with the increase in the concentration of ascorbic acid (table 4). The calibration curve ($y = 0.060x^3 - 0.363x^2 + 0.794x - 0.013$) was determined with peak intensities of the internal standard signal in Mn^{2+} using standard ascorbic acid solution. Hydroxyl radical scavenging activity of *E. thymifolia* was calculated

Table 5. Hydroxyl radical scavenging activity assay of *E. thymifolia*

Sample	Concentration g/ml	ESR signal peak height		Ascorbic acid[a] m*M*	Hydroxyl radical scavenger activity[b], unit/g
		Mn^{2+}	radical		
Water extract	4.83×10^{-1}	52.40	518.40	0.19	0.40
3-*O*-Galloyl-4, 6-(*S*)-HHDP-D-glucose	6.62×10^{-1}	51.60	424.60	0.33	0.50
Rugosin B	–	52.00	527.60	0.17	–
1,3,4,6-Tetra-*O*-galloyl-*β*-D-glucose	–	52.20	648.60	0.01	–

[a] Calibration curve: $y = 0.060x^3 - 0.363x^2 + 0.794x - 0.013$, where y = concentration of ascorbic acid (m*M*), $x = (I_0/I - 1)$; $I_0 = 12.42$ (from table 6); I indicates the average relative peak height, which was the ratio of the peak height of radical/Mn^{2+} in various concentrations of test samples.

[b] 1 unit is the hydroxyl radical scavenging activity of 1 m*M* ascorbic acid in this system.

Table 6. Anti-HSV type 2 activities and cytotoxic effects of fractions and pure compounds of *E. thymifolia* on Vero cells

Compound	Antiviral activity, IC_{50}, μg/ml[b]		Cytotoxicity CC_{50}, μg/ml[c]	Selectivity index[d]	
	XTT	PRA		XTT	PRA
EtOAc fraction	7.72 ± 0.15	7.42 ± 1.63	35.15 ± 5.37	4.55	4.74
n-BuOH fraction	>125	ND	ND	NE	NE
Organic aqueous fraction	>125	ND	ND	NE	NE
Water extracts	>10	ND	ND	NE	NE
3-*O*-Galloyl-4,6-(*S*)-HHDP-D-lucose	4.75 ± 0.46	3.51 ± 0.44	18.13 ± 0.54	3.81	5.17
Rugosin B	>200	ND[e]	ND[e]	NE	NE
Acyclovir	0.18 ± 0.01	0.07 ± 0.01	>200	>1,111	>2,857

Each value represents the mean ± SD of three independent experiments. ND = Not done; NE = not evaluated.

[a] Selectivity index (SI) was the ratio of CC_{50} to IC_{50}.

[b] Antiviral activity was determined by XTT and plaque reduction assays (PRA). 50% inhibition concentration (IC_{50}) was the concentration of fractions and pure compounds of *E. thymifolia* that inhibit 50% HSV-2 growth in the Vero cell line.

[c] Cytotoxicity was determined by XTT assay. 50% cytotoxic concentration (CC_{50}) was the concentration of fractions and pure compounds of *E. thymifolia* that achieved 50% cytotoxicity against Vero cell line.

with the calibration curve. The hydroxyl radical scavenging activity of water extract and 3-*O*-galloyl-4,6-*(S)*-HHDP-*D*-glucose was 0.40 unit/g and 0.50 unit/g, respectively (table 5).

Antiviral Activity

Results on the anti-herpes simplex virus type-2 (HSV-2) of *E. thymifolia* are shown in table 6. Acyclovir, which is commonly used in clinical treatment for HSV infection therapy, served as positive control. The IC_{50} values in XTT and plaque reduction assays for 3-*O*-galloyl-4,6-*(S)*-HHDP-*D*-glucose were 4.75 ± 0.46 and 3.51 ± 0.44 μg/ml, whereas for EtOAc fractions they were 7.72 ± 0.15 and 7.42 ± 1.63 μg/ml, respectively.

Cytotoxicity Assay

Cytotoxicity assay was performed to ensure that the antiviral activity of *E. thymifolia* and acyclovir were not caused by cytotoxic effects of test samples toward the Vero cell line. The CC_{50} values of 3-*O*-galloyl-4,6-*(S)*-HHDP-*D*-glucose, EtOAc fraction and acyclovir against the Vero cell line were 18.13 ± 0.54 , 35.15 ± 5.37 and >200 μg/ml, respectively (table 6).

Selectivity Index

The selectivity index is used to evaluate the safety of a sample when applied in a biological system. As shown in table 6, the SI value of 3-*O*-galloyl-4,6-*(S)*-HHDP-*D*-glucose, EtOAc fraction, and acyclovir in XTT assay was 3.82, 4.55, and >1,111, whereas in plaque reduction assays it was 5.17, 4.74, and >2,857, respectively.

Discussion

Reactive oxygen species (ROS) such as superoxide radicals, hydroxyl radicals, iron-oxygen complexes, hydrogen peroxide and lipid peroxides are generated by several oxidative reactions [32]. Although ROS can enhance the clearance of extrusive microorganisms by the immune system, excess ROS can react with biological molecules such as DNA, proteins and phospholipids and consequently cause a variety of diseases, including ischemia-reperfusion injury, diabetes mellitus, cancer and others [21, 24, 31, 34]. Fortunately, the 'antioxidant' enzymes in our body can convert excess ROS into non-toxic compounds. However, the imbalance in the amount of ROS and 'antioxidant' enzyme is believed to be the main cause of many diseases. This is why the intake of daily foods with antioxidant activity is necessary for a healthy life.

Our studies showed that the extracts and pure compounds of *E. thymifolia* exhibited antioxidant activity. *E. thymifolia* not only inhibited the peroxidation of lipid and the formation of superoxide radicals, but also showed activities in the scavenging of superoxide and hydroxyl radicals. Nevertheless, the hydroxyl radical scavenging activity of *E. thymifolia* was considered to be less potent than its superoxide radical scavenging activity.

Although extracts and pure compounds of *E. thymifolia* exhibited antioxidant activities, only 3-*O*-galloyl-4,6-*(S)*-HHDP-*D*-glucose and EtOAc extract were found to possess anti-HSV-2 activity. Thus, EtOAc extract and 3-*O*-galloyl-4,6-*(S)*-HHDP-*D*-glucose from *E. thymifolia* were concluded to exhibit a broad spectrum of biological activities.

Previous studies demonstrated that viral infection could increase the amount of free radicals in mice and consequently cause pathology [1, 23]. The intake of free radical scavengers improved the survival rate of viral-infected mice [23]. Our studies on *E. thymifolia* revealed that rugosin B and 1,3,4,6-tetra-*O*-galloyl-*β*-*D*-glucose possess a greater potency than 3-*O*-galloyl-4,6-*(S)*-HHDP-*D*-glucose in inhibiting superoxide radical formation and lipid peroxidation. Although rugosin B exhibited a lower IC_{50} than 3-*O*-galloyl-4,6-*(S)*-HHDP-*D*-glucose in superoxide radical scavenging activity, it was 3-*O*-galloyl-4,6-*(S)*-HHDP-*D*-glucose, but not rugosin B and 1,3,4,6-tetra-*O*-galloyl-*β*-*D*-glucose that showed hydroxyl radical scavenging and anti-HSV-2 activities. This preliminary finding suggested that the antiviral activity of 3-*O*-galloyl-4,6-*(S)*-HHDP-*D*-glucose may be primarily related to its hydroxyl radical scavenging activity. Further studies are needed to explain its exact relationship. It was concluded that *E. thymifolia* possesses both antioxidant and anti-HSV-2 activities.

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