Antioxidant and Free Radical Scavenging Activities of *Terminalia chebula*

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Free radicals react with biological molecules and destroy the structure of cells, which eventually causes free-radical induced disease such as cancer, renal failure, aging, *etc*. In this study, 6 extracts and 4 pure compounds of *Terminalia chebula* RETZ. were investigated for anti-lipid peroxidation, anti-superoxide radical formation and free radical scavenging activities. The superoxide radical scavenging of the 4 pure compounds was further evaluated using electron spin resonance (ESR) spectrometry. The results showed that all tested extracts and pure compounds of *T. chebula* exhibited antioxidant activity at different magnitudes of potency. The antioxidant activity of each pure compound was derived from different pathways and was suggested to be specific.

Key words *Terminalia chebula*; antioxidant activity; casuarinin; chebulanin; chebulinic acid; 1,6-di-*O*-galloyl-*β*-D-glucose

Free radicals have been regarded as a fundamental cause of different kinds of diseases. They cause biochemical damage in cells and tissues, which result in several diseases such as arteriosclerosis, ischemia-reperfusion injury, liver disease, diabetes mellitus, inflammation, renal failure, aging, cancer, *etc*.[1,2] Compounds that can scavenge free radicals are thus effective in ameliorating the progress of these related diseases.

Terminalia chebula RETZ. (Combretaceae), a native plant in India and Southeast Asia, is extensively cultivated in Taiwan. Its dried ripe fruit, also called as medicinal terminalia fruit, has traditionally been used to treat various ailments in Asia.[3] In Myanmar, medicinal terminalia fruit is used as a laxative and tonic agent. In China, it is applied as a carminative, deobstruent, astringent and expectorant agent, and also as a remedy for salivating and heartburn. In Indo-China, it is regarded as a purgative agent. In Malaysia, medicinal terminalia fruit is believed to exhibit anti-diarrheic, styptic, antibilious and anti-dysenteric activities.

T. chebula has been reported to exhibit a variety of biological activity, including anticancer,[4] antidiabetic,[5] antimutagenic,[6,7] antibacterial,[8] antifungal,[9] and antiviral[10—12] activities, *etc*. Its methanol and acetone extracts have shown antioxidant activity.[5,13] However no antioxidant activity of casuarinin, chebulanin, chebulinic acid or 1,6-di-*O*-galloyl-*β*-D-glucose, isolated and purified from *T. chebula*, had been reported. This is the first report on the antioxidant activity of pure compounds from *T. chebula*.

Oxidation is believed to be involved in the early stages of carcinogenesis.[14] Compounds that can scavenge excessive free radicals in the body are suggested to hinder the process of carcinogenesis. Since *T. chebula* and chebulinic acid has been reported to exhibit anticancer activity,[4] we were thus interested in studying the antioxidant effect of *T. chebula* and its pure compounds. In this study, 6 extracts and 4 pure compounds of *T. chebula* were evaluated for anti-lipid peroxidation, anti-superoxide radical formation and free radical scavenging activities. The superoxide radical scavenging of the 4 pure compounds was further investigated using electron spin resonance (ESR) spectrometry.

MATERIALS AND METHODS

Plant Material The dried and matured fruits of *T. chebula* were collected from the southern market of Taiwan. These were identified and confirmed with anatomical and morphological techniques by Prof. Chun-Ching Lin (Graduate Institute of Natural Products, College of Pharmacy, Kaohsiung Medical University, Kaohsiung, Taiwan). A voucher specimen was deposited at the Herbarium of the Graduate Institute of Natural Products of Kaohsiung Medical University, Kaohsiung, Taiwan.

Preparation of Samples Four hundred grams dried and ripe fruits of *T. chebula* were hammered into small pieces, followed by extraction with 1 l of MeOH for 24 h. The extract was then filtered and collected. The residual was extracted again with another 1 l of MeOH for another 24 h. The same procedure was repeated two times. The collected MeOH extracts were then combined, concentrated under reduced pressure, and lyophilized until dry. The final yield of MeOH extract was 200 g. Among the 200 g of lyophilized MeOH extract, 100 g were resuspended in 500 ml H_2O. This was then extracted successively with 500 ml $CHCl_3$, 500 ml EtOAc and 500 ml *n*-BuOH. The residual solvent, which includes a H_2O layer and an emulsion layer, was collected and identified as organic aqueous fraction. The solvent of each extract was then removed under reduced pressure, and the resulting extracts were subsequently lyophilized to dry. The final yield of $CHCl_3$, EtOAc, *n*-BuOH and organic aqueous extracts was 2.3, 36.4, 3.6 and 1.4 g, respectively.

On the other hand, another 600 g dried and ripe fruits of *T. chebula* were hammered into small pieces, then extracted with 1 l of warm H_2O for 24 h. The same procedure was repeated another two times. The extracts were then combined, concentrated under reduced pressure, and finally lyophilized to dry. The final yield of the water extracts was 237.0 g.

Besides the extracts, casuarinin, chebulanin, chebulinic acid and 1,6-di-*O*-galloyl-*β*-D-glucose, isolated from *T. chebula*, were also tested in this study. These pure compounds were isolated and their structures were identified as described previously.[15] The structures of these pure compounds are shown in Fig. 1.

Chemicals L(+)-Ascorbic acid, thiobarbituric acid

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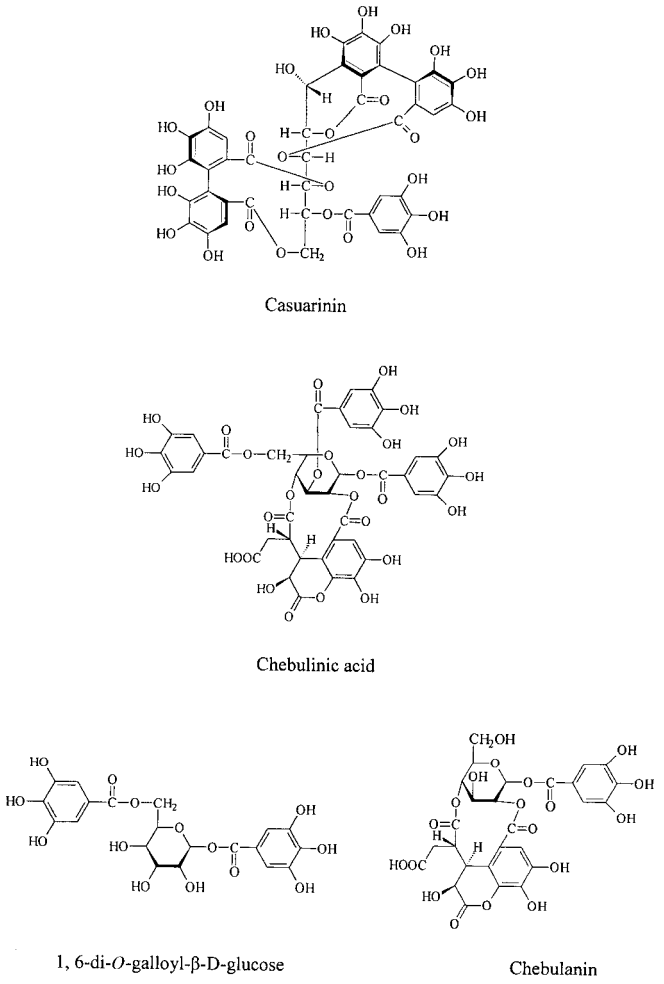

Fig. 1. Structure of Pure Compounds Isolated from *T. chebula*

(TBA), sodium dodecyl sulfate (SDS), cytochrome C, allopurinol, trolox, hypoxanthine, ethylenediaminetetraacetic acid (EDTA), diethylenetriamine pentaacetic acid (DETAPAC) and xanthine were purchased from Sigma Chemical Co. (U.S.A.). Anhydrous iron(II) chloride was purchased from Wako Pure Chemical Ind., Ltd. (Japan). KH_2PO_4 was purchased from Ferak Chemical Co. (Germany). 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO) and xanthine oxidase were purchased from Labotec Co., Ltd. (Tokyo, Japan) and Boehringer Mannheim, respectively.

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

Preparation of Liver Homogenate Liver homogenate was prepared according to the method of Masaol *et al.*[16] with minor modification. Rats weighing between 180 to 230 g were sacrificed. The liver was quickly removed and cut into pieces. The liver sample was then homogenized in 150 mM Tris–HCl (pH 7.2) with a disperser (Ultra-Turrax T25, IKA-Labortechnik) at 20500×***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. The supernatant of liver homogenate was collected, and the amount of protein was determined using a *DC*™ Protein Assay kit (Bio-Rad) according to the protocols recommended by the manufacturer.

Anti-lipid Peroxidation Assay The anti-lipid peroxidation activity of extracts and pure compounds from *T. chebula* was evaluated according to the previously described procedures.[17,18] Briefly, a reaction mixture contained 250 μl of rat liver homogenate (50 mg of protein), 100 μl of Tris–HCl buffer (pH 7.2), 50 μl of 0.1 mM ascorbic acid, 50 μl of 4 mM $FeCl_2$, and then 50 μl of tested extract was mixed in a capped tube and incubated at 37 °C for 1 h. After incubation, 500 μl of 0.1 N HCl, 200 μl of 9.8% SDS, 900 μl de-ionized water and 2 ml of 0.6% TBA was successively added. The mixture was vigorously shaken before it was placed into a boiling water bath (95 °C) for 30 min. After cooling, 5 ml *n*-BuOH was added. The mixture was then centrifuged at 1000×***g*** at a temperature of 25 °C for 25 min to remove flocculent precipitate. The lipid peroxide concentration was determined by MDA-TBA adduct (completion of malondialdehyde with thiobarbituric acid) at 532 nm using a Hitachi U-2000 spectrophotometer. The concentration of the extracts and pure compounds of *T. chebula* that inhibited the production of 50% lipid peroxide (IC_{50}) was calculated as previously described.[19]

Anti-superoxide Formation Assay The anti-superoxide formation activity of *T. chebula* was evaluated by spectrophotometric measurement of the formation of uric acid from the xanthine/xanthine oxidase system.[20,21] The samples were first dissolved with DMSO, then diluted to the desired concentration with 50 mM of KH_2PO_4 (pH=7.8) solution. 50 μl of the sample solution, 400 μl of 0.1 mM xanthine in 50 mM of KH_2PO_4 (pH=7.8) solution, and 530 μl of de-ionized H_2O were added into the tube. The mixture was vigorously shaken. 20 μl of 1 unit xanthine oxidase solution in 1 ml of de-ionized H_2O was then added. After vigorous mixing, the solution was screened for 2 min at 295 nm. The IC_{50} of each sample was calculated from the regression line.

Free Radical Scavenging Activity Assay Free radical scavenging activity was assayed spectrophotometrically by the cytochrome C reduction method as described by McCord and Fridovich[22] and Yu *et al.*[23] When xanthine oxidase converts xanthine to uric acid, the superoxide anion produced would reduce ferricytochrome C to ferrocytochrome C. Since ferrocytochrome C shows a 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 *T. chebula* can be evaluated using the cytochrome C reduction method. Samples were dissolved in DMSO and diluted to desired concentrations with 50 mM of KH_2PO_4 (pH=7.8) solution. Fifty microliters of sample solution, 400 μl of working solution (prepared by mixing 20 ml of 0.1 mM xanthine, 1 ml of 0.1 mM cytochrome C, 1 ml of 0.1 mM EDTA and 1 ml of 50 mM KH_2PO_4 together), 530 μl of de-ionized H_2O and 20 μl of 1 unit xanthine oxidase solution (1 unit of xanthine oxidase in 1 ml of de-ionized H_2O) were mixed vigorously and then screened for 70 s at 550 nm. The IC_{50} of each sample was calculated from the regression line.[19]

Superoxide Anion Scavenging Activity Assay in ESR

The superoxide radical scavenging activity of *T. chebula* was also evaluated using electron spin resonance (ESR) spectrometry. Superoxide radical was generated from the hypoxanthine–xanthine oxidase reaction system and then trapped by DMPO. The product, a spin adduct (DMPO-OOH), was analyzed using an ESR spectrometer.[24—26] Solutions of 2.0 mM hypoxanthine/PBS (A), 5.5 mM DETAPAC (B), various concentrations of tested extracts or superoxide dismutase (SOD) (C) and 0.4 unit/ml of xanthine oxidase/PBS (D) were prepared before use. Solution D was stored in an ice bath to prevent any inactivation of the 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 were then added to the mixed solution to produce DMPO-OOH.

The reaction mixture that was prepared to generate DMPO-OOH was stirred and then transferred into a quartz-analyzing cell. It was later placed into the cavity of an ESR spectrometer (JEOL-JES-FR80, JEOL Ltd., Tokyo, Japan). Forty seconds after the addition of xanthine oxidase, the reaction mixture was analyzed and the relative intensity of the spin adduct (DMPO-OOH) signal was calculated as the ratio of the intensity signal of the radical to that of Mn^{2+}. ESR spectra were recorded at 37 °C with the field set at 335.4± 5.0 mT, modulation frequency at 100 kHz, modulation amplitude at 0.79×0.1 mT, responding time for 0.1 s, sweeping time for 2 min, microwave power at 8.0 mW (9.416 GHz), and receiver gain at 2×100.

RESULTS

The anti-lipid peroxidation, anti-superoxide formation and free radical scavenging activities of 6 extracts and 4 pure compounds of *T. chebula* were evaluated, and the results are summarized in Table 1. The results showed that all tested extracts and pure compounds possessed anti-lipid peroxidation activity at several levels. The IC_{50} values of all tested compounds, except casuarinin, were lower than 9.00 mg/ml. Casuarinin was considered to exhibit weak anti-lipid peroxidation activity when compared with the other tested compounds. The IC_{50} value of casuarinin was 29.67 mg/ml, 13-fold higher than trolox, a reference compound.

When tested for anti-superoxide radical formation activity, all of the tested extracts and pure compounds had IC_{50} values in the range of 0.04—2.42 mg/ml. The noteworthy compound was chebulanin. Chebulanin had an IC_{50} value of 0.04 mg/ml. This IC_{50} value was lower than that of allopurinol, a reference compound. On the other hand, organic aqueous extract, casuarinin, chebulinic acid and 1,6-di-*O*-galloyl-β-D-glucose exhibited weak anti-superoxide radical formation activity, with IC_{50} values at least 15-fold higher than that of allopurinol.

Besides the anti-lipid peroxidation and anti-superoxide formation activities, *T. chebula* was also investigated for its free radical scavenging activity. Results showed that chebulinic acid had the strongest free radical scavenging activity among the tested extracts and pure compounds. The IC_{50} value of chebulinic acid was 0.002 mg/ml. Other tested samples, including, MeOH, $CHCl_3$, EtOAc, *n*-butanol, water extracts and casuarinin, were also concluded to possess free radical scavenging activity with IC_{50} values in the range of 0.004—0.009 mg/ml. The free radical scavenging activity of organic aqueous extract and chebulanin was moderate, and that of 1,6-di-*O*-galloyl-β-D-glucose was weak.

Table 1. Anti-lipid Peroxidation, Anti-superoxide Radical Formation, and Free Radical Scavenging Activities of *T. chebula in Vitro*

Extracts and pure compounds	IC_{50} (mg/ml)[a]		
	Anti-lipid peroxidation activity	Anti-superoxide radical formation activity	Free radical scavenging activity
MeOH extract	4.44	0.48	0.005
$CHCl_3$ extract	5.39	0.55	0.006
EtOAc extract	4.05	0.50	0.004
n-Butanol extract	4.35	0.88	0.008
Organic aqueous extract	8.97	2.42	0.021
Water extract	4.80	0.73	0.009
Casuarinin	29.67	1.06	0.006
Chebulanin	3.96	0.04	0.031
Chebulinic acid	7.27	1.17	0.002
1,6-Di-*O*-galloyl-β-D-glucose	ND[b]	1.30	0.161
Allopurinol	ND[b]	0.06	ND[b]
Trolox	2.14	ND[b]	ND[b]

a) Each value represents the mean of six tests. *b*) ND=Not done.

Table 2. Superoxide Radical Scavenging Activity of *T. chebula* in ESR

Sample[a]	IC_{50} (mg/ml)[b]
Casuarinin	2.82×10^{-3}
Chebulanin	19.63×10^{-3}
Chebulinic acid	18.81×10^{-3}
1,6-Di-*O*-galloyl-β-D-glucose	9.03×10^{-3}

a) The experimental concentrations of casuarinin, chebulanin, chebulinic acid and 1,6-di-*O*-galloyl-β-D-glucose were 5.93×10^{-6}, 4.70×10^{-5}, 4.50×10^{-5}, and 2.00×10^{-5} g/ml, respectively. *b*) A linear calibration curve ($y=0.235x-0.062$) was established using a standard SOD solution, and the superoxide radical scavenging activity of *T. chebula* was calculated as described in the text. The IC_{50} indicates the amount of test sample that caused 50% inhibition of the ESR signal intensity of DMPO-OOH.

The antioxidant activity of pure compounds of *T. chebula* was further evaluated by the ESR spin-trapping technique, a powerful tool in investigating superoxide radical scavenging activity. When DMPO was added to a solution of the hypoxanthine-xanthine oxidase reaction system, the spin adduct DMPO-OOH was formed. The relative peak height, the ratio of the peak height of radical/Mn^{2+}, was calculated. It was observed that the signal intensity of DMPO-OOH was decreased with the addition of SOD in a dose-dependent manner. This is because SOD can inhibit the reaction between $O_2^{\cdot-}$ and DMPO. The linear calibration curve ($y=0.235x-0.062$) was then established according to the relative peak intensities of radical/Mn^{2+} using a standard SOD solution (0.00—18.83 units/ml in concentration). When pure compounds of *T. chebula* were added into the hypoxanthine–xanthine oxidase reaction system, the signal intensity of DMPO-OOH was recorded. The superoxide radical scavenging activity of *T. chebula* was calculated by comparison of the average relative peak height between *T. chebula* and standard SOD. Our results show that all tested pure compounds exhibited the inhibition of DMPO-OOH signal intensities (Table 2, Fig. 2). The strength of superoxide radical scavenging activity was in the order of casuarinin>1,6-di-*O*-galloyl-β-D-glucose>chebulinic acid>chebulanin, with the IC_{50} values

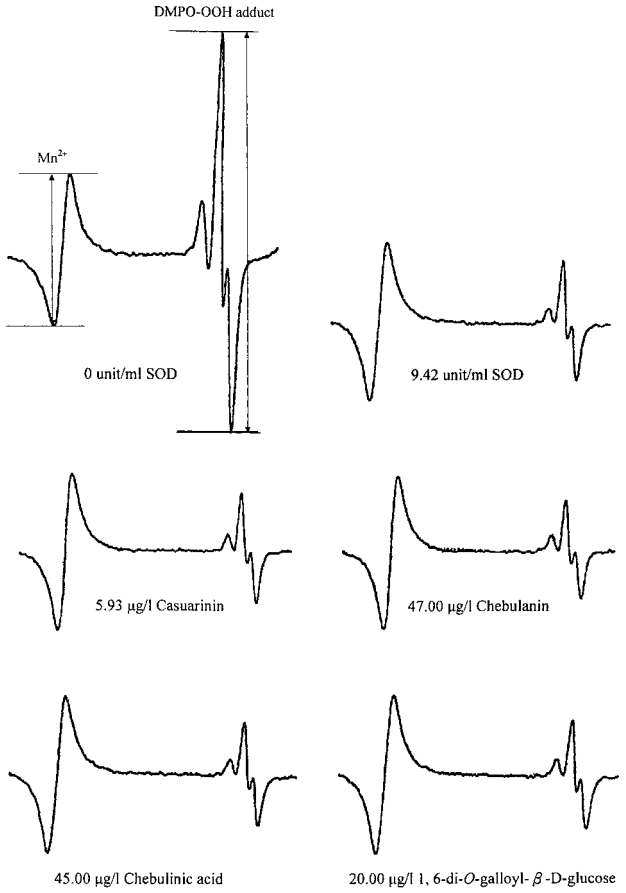

Fig. 2. The Inhibitory Effect of SOD and Pure Compounds Isolated from *T. chebula* on ESR Signals of Superoxide Radical

of 2.82, 9.03, 18.81 and 19.63 μg/ml, 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.[14] Although ROS can help the immune system clean out extrusive microorganisms, excessive ROS can also react with biological molecules such as DNA, proteins and phospholipids, and eventually cause oxidative damage in tissue and free radical-related diseases such as inflammation, heart disease, diabetes, gout, cancer, *etc.*[27] For aerobic organisms, the major system of defense against oxidative damage is the use of "antioxidant" enzymes to convert excessive ROS into non-toxic compounds. An imbalance between the amount of ROS and 'antioxidant' enzymes is a problem for our health. This is why the daily intake of foods with antioxidant activity is necessary.[28,29]

T. chebula is a medicinal plant traditionally used in Southeast Asia, India, China and Taiwan. It has been used to treat different ailments for a long time. There is a belief that the causation and progression of some of these ailments, in some ways, are related to damage caused by ROS. For example, oxidants have been considered to be involved in the early stages of carcinogenesis,[14] whereas oxygen free radicals have played an important role in the development of the long-term complications of diabetes.[30] Although the intake of antioxidants may not completely cure the disorders, it can reduce the oxidative stress in our body and consequently ameliorate the progress of related diseases.

In this study, 6 extracts and 4 pure compounds of *T. chebula* were evaluated for their antioxidant activity. All of them were demonstrated to exhibit antioxidant activity at different magnitudes of potency. Recently, many biological activities of *T. chebula*, such as anticancer,[4] antidiabetic,[5] antimutagenic,[6,7] antibacterial,[8] antifungal,[9] and antiviral[10—12] activities, *etc.* have been reported in the literature. These biological activities, in some terms, may be partially related to antioxidant activity. For example, the antioxidant activity of the methanolic extract of *T. chebula* has been suggested to play a role in the relief of long-term complications in diabetic rats by reducing the oxidative stress.[5] Also, the antimutagenic and anticancer activities of *T. chebula* may account for its antioxidant activity because antioxidant agents can hinder the process of carcinogenesis.[14] Since the intake of free radical scavengers will improve the survival rate of influenza-infected mice,[31] the antiviral activity of *T. chebula* was thus possibly related to its antioxidant activity, too.

When evaluated for antioxidant activity, an interesting result was observed among the tested pure compounds. It was found that tested pure compounds, in some terms, were specific in their antioxidant activity. For example, although casuarinin was active in scavenging free radicals, its anti-superoxide formation and anti-lipid peroxidation activities were moderate or weak. For chebulanin, the inhibitory effects on the peroxidation of lipids and the formation of superoxide radicals were more significant than that on the scavenging of free radicals. Also, it was considered that the antioxidant activity of chebulinic acid was primarily derived from its free radical scavenging activity than by anti-superoxide formation or anti-lipid peroxidation activities.

In conclusion, all tested extracts and pure compounds of *T. chebula* showed antioxidant activity at different magnitudes of potency. The antioxidant activity of each pure compound was derived from different pathways and was suggested to be specific in some term. Some previously reported biological activities of *T. chebula* were possible partially related to its antioxidant activity. However, their exact relationship needs further investigation.

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