Antioxidant Activities of *Physalis peruviana*

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Physalis peruviana **(PP) is a widely used medicinal herb for treating cancer, malaria, asthma, hepatitis, dermatitis and rheumatism. In this study, the hot water extract (HWEPP) and extracts prepared from different concentrations of ethanol (20, 40, 60, 80 and 95% EtOH) from the whole plant were evaluated for antioxidant activities. Results displayed that at 100 μg/ml, the extract prepared from 95% EtOH exhibited the most potent inhibition rate (82.3%) on $FeCl_2$–ascorbic acid induced lipid peroxidation in rat liver homogenate. At concentrations 10—100 μg/ml, this extract also demonstrated the strongest superoxide anion scavenging and inhibitory effect on xanthine oxidase activities. In general, the ethanol extracts revealed a stronger antioxidant activity than α-tocopherol and HWEPP. Compared to α-tocopherol, the IC_{50} value of 95% EtOH PP extract was lower in thiobarbituric acid test (IC_{50}=23.74 μg/ml *vs.* 26.71 μg/ml), in cytochrome *c* test (IC_{50}=10.40 μg/ml *vs.* 13.39 μg/ml) and in xanthine oxidase inhibition test (IC_{50}=8.97 μg/ml *vs.* 20.68 μg/ml). The present study concludes that ethanol extracts of PP possess good antioxidant activities, and the highest antioxidant properties were obtained from the 95% EtOH PP.**

Key words *Physalis peruviana*; lipid peroxidation; antioxidant activity; cytochrome *c*; xanthine oxidase

Free radicals have been regarded as the fundamental cause of different kinds of diseases, including aging, coronary heart disease, inflammation, stroke, diabetes mellitus, rheumatism, liver disorders, renal failure and cancer.[1—3] Reactive oxygen species (ROS) comprise free radicals such as $\cdot O_2^-$ (superoxide anion), $\cdot$OH (hydroxyl radical), H_2O_2 (hydrogen peroxide) and $^1O_2$ (singlet oxygen), which can cause cellular injuries and also initiate peroxidation of polyunsaturated fatty acids in biological membranes.[4,5] The tissue injury caused by ROS include DNA damage,[5,6] protein damage[7] and oxidation of enzymes[8] in the human body. Antioxidants such as α-tocopherol is capable of mitigating free radical damage and scavenging ROS.[9,10]

Enzymatic antioxidants include superoxide dismutase (SOD), catalase and glutathione peroxidase (GPX), whereas non-enzymatic antioxidants comprise α-tocopherol, β-carotene, carotenoids, chlorophylls, flavonoids, tannin and certain micronutrients (*e.g.* zinc and selenium).[11—13] The present of these antioxidants in the cellular system are known to prevent oxidative damage. Thus, a number of antioxidants derived from plants such as *Ginkgo biloba*,[14] *Prunella vulgaris*,[15] *Therminalia chebula*,[16] *Cinnamomum cassia*.[17] have been widely studied. Dietary intake of antioxidants has been increasingly accepted as a strategy for maintaining a healthy life.

P. peruviana is a medicinal plant widely used in folk medicine as anticancer, antimycobacterial, antipyretic, immunomodulatory, and for treating diseases such as malaria, asthma, hepatitis, dermatitis, diuretic and rheumatism.[18—20] Hot water extract of *P. peruviana* (HWEPP) is often used to prepare health beverages. However, it remains unknown if HWEPP and extracts prepared from different concentrations of ethanol possess antioxidant activity. In this study, our objective was to evaluate the antioxidant activities of HWEPP and extracts prepared from 20, 40, 60, 80 and 95% ethanol of whole plant of *P. peruviana*. Its antioxidant activities were also compared with a well known natural antioxidant, α-tocopherol.

MATERIALS AND METHODS

Chemicals L-(+)-Ascorbic acid, thiobarbituric acid (TBA), xanthine, xanthine oxidase and cytochrome *c* were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). α-Tocopherol, dimethylsulphoxide (DMSO) and ferrous chloride were obtained from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals used were of analytical grade.

Animals Male Wistar rats, age 4—6 weeks old, were obtained from the animal center of the National Cheng Kung University, Tainan, Taiwan. They were housed in an air-conditioned room with temperature maintaining at 22±3 °C and humidity at 55±5%. Animals were fed a standard laboratory diet and tap water ad libitum throughout the experimental period. They were used for $FeCl_2$–ascorbic acid induced lipid peroxidation study.

Plant Materials The plant of *Physalis peruviana* L. (PP) of the family Solanaceae was obtained from Tainan District Agricultural Research and Extension Station, Taiwan. The authenticity of the plant species was confirmed by Professor CC Lin of Graduate Institute of Natural Products, Kaohsiung Medical University, Taiwan. The whole plants were dried and ground to sawdust form, which was then kept in an air-tight brown bottle until use.

Preparation of Aqueous Extract One hundred grams of sample was extracted with 1 l of boiling water for 1 h. The extract was filtered while the residue was re-extracted under the same conditions twice. The filtrates collected were combined and evaporated to dryness under vacuum. The average yield obtained for HWEPP was 20.7%.

Preparation of Ethanol Extract One hundred grams of *P. peruviana* sawdust was soaked with 1 l of 20, 40, 60, 80

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and 95% ethanol at room temperature for 6 d. The extract was filtered with filter paper (Advantec No. 1, Japan) while the residue was further extracted under the same conditions twice. The filtrates collected from three separate extractions were combined and evaporated to dryness under vacuum. The average yield obtained for 20% (20EtOH-PP), 40% (40EtOH-PP), 60% (60EtOH-PP), 80% (80EtOH-PP) and 95% (90EtOH-PP) ethanol extracts (EEPP) were 7.3, 17.7, 20.7, 24.1 and 24.0%, respectively.

Preparation of Liver Homogenate Liver homogenate was prepared according to a previously described method with minor modification.[21] Briefly, rats weighing 180—230 g were sacrificed and the liver was quickly removed. The liver sample was then cut into small pieces and homogenized in 150 mM Tris–HCl buffer (pH 7.2) with a disperser (PT1300D, Kinematica) 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. Supernatant of the liver homogenate was collected and the amount of protein was determined by the method of Lowry *et al.*[22]

$FeCl_2$-Ascorbic Acid Stimulated Lipid Peroxidation in Rat Liver Homogenate The effect of PP extracts on $FeCl_2$–ascorbic acid induced lipid peroxidation in rat liver homogenate was conducted according to the modified methods as described by Kimura *et al.*[23] and Wong *et al.*[24] A mixture containing 0.5 ml of liver homogenate, 0.1 ml of Tris–HCl buffer (pH=7.2), 0.05 ml of 0.1 mM ascorbic acid, 0.05 ml of 4 mM $FeCl_2$ and 0.05 ml of various concentrations of EEPP, HWEPP or α-tocopherol. It was then incubated for 1 h at 37 °C. After incubation, 0.9 ml of distilled water and 2 ml of 0.6% TBA were added and then shaken vigorously. The mixture was heated for 30 min in a boiling water bath at 100 °C. After cooling, 5 ml of *n*-butanol was added and the mixture was then shaken vigorously. The *n*-butanol layer was separated by centrifugation at 3000×***g*** for 10 min. The supernatant was collected and measured spectrophotometrically at 532 nm.

Cytochrome *c* Test Enzymatic formation of superoxide anions was assayed by the reduction of cytochrome *c* method.[25] Ten milligrams of samples were dissolved in 1 ml of distilled water or DMSO and then diluted with 50 mM phosphate buffer (pH=7.8) to various concentrations (10 to 100 μg/ml), followed by adding 0.07 units/ml of xanthine oxidase, 100 μM of xanthine and 50 μM of cytochrome *c* to the samples. All samples were incubated for 3 min at room temperature and then measured spectrophotometrically at 550 nm.

Xanthine Oxidase Inhibition Test Xanthine oxidase activity was estimated by the formation of uric acid from xanthine.[26] Ten milligrams of samples were dissolved in 1 ml distilled water or DMSO, and then diluted with 50 mM KH_2PO_4 buffer (pH=7.8) to various concentrations (10 to 100 μg/ml). After 100 μM of xanthine and 20 μl of xanthine oxidase (0.4 units) were added, samples were incubated for 3 min at room temperature. Superoxide formation was counted by spectrophotometric measurement of uric acid production at 295 nm.

Statistical Analysis Data are presented as means±S.D. IC_{50} value of each sample was calculated. Values were evaluated by one way analysis of variance (ANOVA), followed by *post hoc* Duncan's multiple range tests.

RESULTS

Anti-Lipid Peroxidation Activity The inhibitory effect of extracts on $FeCl_2$–ascorbic acid induced malondialdehyde (MDA) production in rat liver homogenate is shown in Table 1. Results showed that the inhibition of MDA formation increases with increasing concentrations of PP extracts and α-tocopherol. At concentrations 10—100 μg/ml, all extracts displayed an anti-lipid peroxidation activity, with an inhibition rate varies from 2.0 to 82.3%. At concentration 100 μg/ml, the formation of MDA was inhibited by 82.3% for 95EtOH-PP and 25.0% for HWEPP.

In the thiobarbituric acid test, the IC_{50} values of 80EtOH-PP and 95EtOH-PP were 23.11 and 23.74 μg/ml, respectively, which were lower than α-tocopherol (26.71 μg/ml) (Table 2). The potency of anti-lipid peroxidation activity was in the order of 80EtOH-PP>95EtOH-PP>α-tocopherol>40EtOH-PP, 60ETOH-PP>20EtOH-PP>HWEPP. Based on the IC_{50} values, 80EtOH-PP and 95EtOH-PP demonstrated the best inhibitory effect against lipid peroxidation.

Free Radical Scavenging Activity The results on the effect of different concentrations of PP extracts on superoxide anions are shown in Table 3. The scavenging effect was found to increase with increasing concentration of PP extracts. At concentrations 10—100 μg/ml, they were shown to possess a scavenging activity ranging from 0.0 to 100.0%. The 95EtOH-PP exhibited a scavenging effect of 49.2% at 10 μg/ml, 99.3% at 50 μg/ml, and 100.0% at 100 μg/ml, which were more active than α-tocopherol at the same concentrations.

Table 1. Inhibitory Effect of Hot Water Extract (HWEPP) and Extracts of Different Ethanol Concentrations (20, 40, 60, 80 and 95% EtOH) Prepared from *P. peruviana* (PP) on $FeCl_2$–Ascorbic Acid Induced MDA Production in Rat Liver Homogenate

Samples	Concentration (μg/ml)	MDA[a] (nM/mg protein)	Inhibition rate[b] (%)
$FeCl_2$-AA	—	50.0±1.5	—
Control	—	20.0±0.8	—
HWEPP	10	49.0±1.0	2.0[g]
20EtOH-PP	10	42.6±0.9	24.7[f]
40EtOH-PP	10	36.2±2.3	46.0[b]
60EtOH-PP	10	38.0±1.5	40.0[d]
80EtOH-PP	10	35.5±1.0	48.3[a]
95EtOH-PP	10	37.0±3.0	43.3[c]
α-Tocopherol	10	38.5±0.5	38.3[e]
HWEPP	50	45.0±1.0	16.7[f]
20EtOH-PP	50	38.0±0.7	40.0[e]
40EtOH-PP	50	35.0±1.2	50.0[d]
60EtOH-PP	50	35.0±2.5	50.0[d]
80EtOH-PP	50	33.8±1.3	54.0[c]
95EtOH-PP	50	31.2±1.0	62.7[b]
α-Tocopherol	50	30.0±0.1	66.7[a]
HWEPP	100	42.5±0.9	25.0[f]
20EtOH-PP	100	36.0±1.5	46.7[e]
40EtOH-PP	100	34.0±0.5	53.3[d]
60EtOH-PP	100	33.6±2.0	54.7[d]
80EtOH-PP	100	27.5±1.8	75.0[c]
95EtOH-PP	100	25.3±0.7	82.3[b]
α-Tocopherol	100	21.2±0.9	96.0[a]

a) MDA data were presented as means±S.D. (n=5). *b*) The inhibitory rates within the column of same concentration with the different superscript letters were significantly different at $p<0.05$ as analyzed by Duncan's multiple range tests.

Table 2. IC_{50} Values of Antioxidant Activities of Hot Water Extract (HWEPP) and Extracts of Different Ethanol Concentrations (20, 40, 60, 80 and 95% EtOH) Prepared from *P. peruviana* (PP)

Samples	IC_{50} values (μg/ml)		
	Thiobarbituric acid test	Cytochrome *c* test	Xanthine oxidase inhibition test
HWEPP	>100	>100	>100
20EtOH-PP	>100	>100	63.93
40EtOH-PP	50.00	55.85	30.02
60EtOH-PP	50.00	52.52	37.79
80EtOH-PP	23.11	27.34	26.00
95EtOH-PP	23.74	10.40	8.97
α-Tocopherol	26.71	13.39	20.68

Table 3. Superoxide Scavenging Activity of Hot Water Extract (HWEPP) and Extracts of Different Ethanol Concentrations (20, 40, 60, 80 and 95% EtOH) Prepared from *P. peruviana* (PP)

Samples	Concentration (μg/ml)	Scavenging rate (%)[a)]
HWEPP	10	0.0 ± 0.0^{f}
20EtOH-PP	10	0.0 ± 0.0^{f}
40EtOH-PP	10	8.3 ± 0.2^{e}
60EtOH-PP	10	20.1 ± 0.5^{d}
80EtOH-PP	10	38.0 ± 0.9^{c}
95EtOH-PP	10	49.2 ± 0.1^{a}
α-Tocopherol	10	47.2 ± 0.3^{b}
HWEPP	50	23.8 ± 0.6^{g}
20EtOH-PP	50	30.2 ± 1.0^{f}
40EtOH-PP	50	43.6 ± 0.1^{e}
60EtOH-PP	50	48.0 ± 1.9^{d}
80EtOH-PP	50	65.7 ± 1.2^{c}
95EtOH-PP	50	99.3 ± 0.1^{a}
α-Tocopherol	50	80.2 ± 0.6^{b}
HWEPP	100	49.0 ± 0.5^{e}
20EtOH-PP	100	48.7 ± 0.8^{e}
40EtOH-PP	100	98.3 ± 1.0^{b}
60EtOH-PP	100	87.7 ± 2.5^{d}
80EtOH-PP	100	95.7 ± 3.1^{c}
95EtOH-PP	100	100.0 ± 0.1^{a}
α-Tocopherol	100	100.0 ± 0.0^{a}

a) Data were presented as the percentage of free radical scavenging, means±S.D. (n=3); values within the column of same concentration with the different superscript letters were significantly different at $p<0.05$ as analyzed by Duncan's multiple range tests.

In the cytochrome *c* test, the 95EtOH-PP (IC_{50} value= 10.40 μg/ml) exhibited the highest scavenging effect on superoxide anions (Table 2). On the other hand, HWEPP and 20EtOH-PP exhibit a weak superoxide radical scavenging activity.

Inhibitory Effect On Xanthine Oxidase Activity All extracts exhibit an inhibitory effect on the xanthine oxidase-induced superoxide formation in a concentration-dependent manner (Table 4). At concentrations 10—100 μg/ml, PP extracts inhibited the xanthine oxidase activity with an inhibition rate ranging from 6.5 to 100.0 % ($p<0.05$). At concentrations 10—100 μg/ml, the xanthine oxidase inhibition effect of all samples was in the order of 95EtOH-PP>α-tocopherol>80EtOH-PP>60EtOH-PP>40EtOH-PP>20EtOH-PP>HWEPP.

In the xanthine–xanthine oxidase system, the IC_{50} value of 95EtOH-PP was 8.97 μg/ml, which is 2.4 fold more active than α-tocopherol (20.68 μg/ml) (Table 2), suggesting that 95EtOH-PP was a potent xanthine oxidase inhibitor.

Table 4. Xanthine Oxidase Inhibition Activity of Hot Water Extract (HWEPP) and Extracts of Different Ethanol Concentrations (20, 40, 60, 80 and 95% EtOH) Prepared from *P. peruviana* (PP)

Samples	Concentration (mg/ml)	Scavenging rate (%)[a)]
HWEPP	10	6.5 ± 0.5^{f}
20EtOH-PP	10	20.0 ± 0.0^{e}
40EtOH-PP	10	31.1 ± 2.0^{d}
60EtOH-PP	10	20.1 ± 0.5^{e}
80EtOH-PP	10	38.0 ± 0.9^{c}
95EtOH-PP	10	59.1 ± 0.8^{a}
α-Tocopherol	10	40.6 ± 0.2^{b}
HWEPP	50	40.0 ± 0.8^{e}
20EtOH-PP	50	40.0 ± 1.5^{e}
40EtOH-PP	50	68.9 ± 2.0^{c}
60EtOH-PP	50	63.1 ± 1.0^{d}
80EtOH-PP	50	68.0 ± 2.5^{c}
95EtOH-PP	50	100.0 ± 0.0^{a}
α-Tocopherol	50	75.9 ± 0.6^{b}
HWEPP	100	46.0 ± 1.0^{e}
20EtOH-PP	100	75.9 ± 2.9^{d}
40EtOH-PP	100	78.0 ± 1.5^{c}
60EtOH-PP	100	77.4 ± 0.8^{c}
80EtOH-PP	100	90.7 ± 2.0^{b}
95EtOH-PP	100	100.0 ± 0.0^{a}
α-Tocopherol	100	100.0 ± 0.0^{a}

a) Data were presented as the percentage of inhibition on xanthine oxidase activity, means±S.D. (n=3); values within the column of same concentration with the different superscript letters were significantly different at $p<0.05$ as analyzed by Duncan's multiple range tests.

DISCUSSION

Excessive ROS can react with biological molecules such as DNA, proteins, phospholipids, and eventually cause oxidative damage in tissue and resulted in free radical-related diseases such as inflammation, heart disease, diabetes, gout and cancer.[27)] Aerobic organisms are protected from ROS toxicity by their natural antioxidant defense system involving enzymatic and non-enzymatic mechanisms. An imbalance between the amount of ROS and the antioxidant defense system could lead to an appearance of health problem. Thus, it is why the daily intake of foods or medicated diet or heath drink with antioxidant activity is important.[6,28)] In this study, six extracts of PP and α-tocopherol were examined for their antioxidant activities. EEPP was shown to possess an excellent antioxidant activity in enzymatic and nonenzymatic liver tissue oxidative systems.

It was reported that Fe^{2+} and ascorbic acid stimulated lipid peroxidation in rat liver microsomes and mitochondria.[23)] α-Tocopherol is a natural antioxidant, which functions as a free-radical quencher in biological cells and localizes within the phospholipid bilayer of cell membranes to protect against biological lipid peroxidation.[29)] Tocopherols were also reported to decrease atherosclerosis and delay death from myocardial infarction, this therapeutic benefit was presumably derived from the inhibition of lipid peroxidation.[30)] The health benefits of certain natural medicines have been explained by their ability in preventing free radical damage to cell membranes through reducing the level of lipid peroxides.

In this study, the 80EtOH-PP and 95EtOH-PP exhibited the best antioxidant action in the rat liver homogenate model system. Although the bioactive components present in these extracts are unknown, it was reported that the trihydroxy steroidal lactone (*i.e.* withaperuvin E) isolated from the roots of PP possess protective effect against lipid peroxidation.[31)]

Compared to SOD, the inhibitory rate of xanthine oxidase activity of 10 μg/ml 95EtOH-PP (49.20%) and 10 μg/ml α-tocopherol (47.20%) was stronger than 10 units/ml SOD (13.00%) but inferior than 100 units/ml (88.46%) and 300 units/ml (93.90%) of SOD. It has reported that xanthine oxidase inhibitors may be useful for the treatment of hepatic disease and gout.[32)] In this study, the 95EtOH-PP displayed the most potent activity in inhibitory the xanthine oxidase activity, suggesting that this extract is an excellent xanthine oxidase inhibitor.

Enzymatic formation of superoxide anions was estimated by reduction of cytochrome *c*. Xanthine oxidase converts hypoxanthine to xanthine and then xanthine to uric acid in the presence of molecular oxygen to yield superoxide anion and hydrogen peroxide. These free radicals can directly reduced ferri-cytochrome *c* to ferro-cytochrome *c*.[14)] Hence, phytochemicals or extracts with inhibitory effect on superoxide anion regeneration by the enzymatic pathway would be beneficial in preventing ischaemia and edema.[33)]

The inhibitory effect of 95EtOH-PP on the lipid peroxidation, superoxide anion regeneration and xanthine oxidase activity were more potent than other extracts. This observation suggests that ethanol PP extracts, particularly the extract of 95EtOH-PP, could be prepared into wines or Chinese medicated diets and be consumed daily in life for maintaining good health. The strong antioxidant activities demonstrated by the ethanol extracts of PP may be contributed by the flavonoids and other yet to be discovered antioxidative compounds.[34,35)] Plants such as *Caesalpinia sappan* heartwood and *Vaccinium berries*, containing flavonoids and phenolics are known to possess strong antioxidant properties.[36,37)]

In conclusion, extracts of PP prepared with different solvent conditions possessed a different magnitude of antioxidant potency. Compared to the aqueous extract, ethanol extracts exhibited a better antioxidant activity, with 95EtOH-PP showed the most potent antioxidant and free radical scavenging activities. The 95EtOH-PP also exhibited the higher antioxidant action in the rat liver homogenate model system. Therefore, ethanol extracts may be used as a potential dietary antioxidant to retard aging and preventing diseases caused by ROS or ameliorating oxidative damage in tissue. The active components and antioxidative mechanism(s) of action of PP ethanol extracts warrant further studies in both *in vitro* and *in vivo* models.

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