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Protective effect of makrut lime leaf (*Citrus hystrix*) in HepG2 cells: Implications for oxidative stress

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ABSTRACT: The objective of the present study was to examine fresh and processed (boiled or fried) leaves of *Citrus hystrix* in terms of total phenolic content, malondialdehyde content, 1,1-diphenyl-2-picrylhydrazyl (DPPH), and hydroxyl radicals scavenging activities, the baseline levels of glutathione *S*-transferase (GST), superoxide dismutase (SOD), and catalase in HepG2 cells. The results indicated that fresh *C. hystrix* leaves possess the higher hydroxyl radical scavenging activity (22 mg/ml). Pretreatment of *C. hystrix* leaf extracts decreases GST, SOD, or catalase activity induced by H_2O_2, rendering them unsuitable for cancer chemoprevention, superoxide scavenging, or hydrogen peroxide detoxification. A linear relation between the total phenolic content and DPPH or hydroxyl radical activities of the extract was not observed. This study showed that *C. hystrix* leaf may exert its antioxidative stress by scavenging hydroxyl radicals and inhibiting lipid peroxidation that causes oxidative damage to HepG2 cells.

KEYWORDS: antioxidant enzyme, hydroxyl radical, lipid peroxidation, phenolics

INTRODUCTION

Oxidative stress, the consequence of an imbalance of prooxidants and antioxidants in the organism, in which oxygen free radicals such as superoxide anions cause oxidative damage, is rapidly gaining recognition as a key mechanism of chronic diseases. Oxygen free radicals, created through aerobic metabolism, are mostly removed by endogenous antioxidants such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT)[1]. Biological specimens containing a mixture of thiobarbituric acid reactive substances (TBARS) include lipid peroxides and aldehydes, both of which increase during oxidative stress. Lipid hydroperoxides formed during oxidative lipid damage leads to dysfunction of membrane-bound receptors. One such byproduct of lipid peroxidation is malondialdehyde (MDA). Exogenous antioxidants such as vitamin C, E, and β-carotene prevent the cascade of oxidative reactions by combining with free radicals[2,3].

Reactive oxygen species (ROS) have been implicated in the etiology of chronic diseases such as atherosclerosis and cancers. A dynamics exists between ROS generation and antioxidant systems within cells. If an imbalance in favour of ROS occurs, oxidative damage to all cell targets (DNA, lipids, proteins) can be aggravated[4]. The ROS H_2O_2 has been reported to generate DNA damage, induce chromosomal aberrations, cause gene mutations, and break single-strand DNA[5]. H_2O_2 may act, via a Fenton-type reaction, to form hydroxyl radicals, which are highly reactive, destructive, and result in direct DNA damage.

HepG2 cells, a human hepatoma cell line, are considered to be a good model for studying in vitro xenobiotic metabolism and toxicity to the liver since they retain many of the specialized functions which characterize normal human hepatocytes[6]. In particular, HepG2 cells retain the activities of phase I, phase II, and antioxidant enzymes ensuring that they constitute a good tool to study cytoprotective, genotoxic, and antigenotoxic effects of compounds[7,8].

Diet plays a major role in the environmental control of oxidative stress: fruits, vegetables, and red wine decrease oxidative stress, whereas the occidental diet, characteristically rich in fats, induces oxidative stress[9]. Thai food is rich in herbs and spices such as makrut lime leaves which contain a large quantity of polyphenols[10]. Bioactive compounds previously identified in *Citrus hystrix* include glyceroglycolipids[11], flavonoids[12], and α-tocopherol[13]. The leaf of *C. hystrix* (Rutaceae) is commonly used as a condiment in various Thai dishes especially in sweet green curry (*kaeng khieow waan*) and the popular sour and spicy shrimp soup (*tom yam kung*) (boiled processing), *khao yam* (raw), and herbal fried bean (fried processing).

It is known that cooking processes bring about

a number of changes in physical characteristics and chemical composition of vegetables[14]. However, very little information is available in the literature regarding the effect of cooking on the baseline levels of SOD, GPx, CAT, and MDA in the liver cancer cell line, HepG2. In the present study, we examine the effect of cooking styles of *C. hystrix* leaf on the antioxidant status of the HepG2 cell line.

MATERIALS AND METHODS

Chemicals

Dulbecco's modification of Eagle's medium (DMEM) was purchased from HyClone (Logan, UT). Penicillin, streptomycin, bovine serum albumin were purchased from Gibco (Paisley, UK). 1,1-diphenyl-2-picrylhydrazyl (DPPH), Folin-Ciocalteu's phenol reagent 2N, 2,2 azinobis (3-ethylbenzothiazoline 6-sulphonate), reduced glutathione (GSH; CAS no. 70-18-8), 1-chloro-2,4-dinitrobenzene (CDNB; CAS no. 97-00-7), catalase assay kit (CAT100), were purchased from Sigma-Aldrich Co. (St Louis, MO). An SOD activity assay kit was purchased from Fluka AG (Buchs, Switzerland).

Preparation of *C. hystrix*

Makrut lime leaves were purchased from a local market. After the stems were removed, the leaves were washed with tap water several times and soaked in sodium lauryl ether sulphate 7% (w/w) for 20 min to remove adhering contaminants and then dried by air. Then, 1200 g was taken and divided into three equal portions. One portion was retained raw, the others were cooked using two different methods (boiling and frying). Makrut lime leaf was boiled for 10 min in boiling water at 100 °C. The samples were drained, blended, and then freeze dried. The fresh leaves were deep-fried in palm oil for 1 min at 145 °C. Both raw and cooked samples were stored at −80 °C and then freeze dried. After freeze drying, the sample was ground in a coffee blender and stored at −18 °C until analysis.

Preparation of *C. hystrix* extract

Extracts were prepared using a modification of the method of Rose, Ong, and Whiteman[15]. In brief, 100 mg of freeze-dried tissue were placed in a 50 ml polypropylene tube, hydrated with 2.0 ml of deionized water, homogenized for 15 s using a sonicator, and left at room temperature for 1 h with occasional stirring. Boiling 700 ml/l methanol (3.0 ml) were added to the mix and incubated for a further 15 min at 70 °C. The mixture was cooled to room temperature, and centrifuged at 1000g for 5 min. After centrifugation, 1 ml aliquots were removed and vacuum condensed to 200 µl volumes. The resultant concentrates were filtered through sterile non-pyrogenic filters (0.2 µm, Millipore) and stored at −70 °C prior to testing. Extracts gave an equivalent concentration of 100 mg/ml for each sample.

Cell culture and treatment

HepG2 cells (hepatocellular carcinoma cell line) obtained from the American Type Culture Collection were maintained in culture in 75 cm^2 polystyrene flasks (Falcon) with DMEM containing 10% FBS and 100 µg/ml penicillin-streptomycin at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Cultures (2.5×10^6 cells) were grown for 3 days in complete DMEM until they reached the post-confluence stage. Cells were incubated with 4 mg/ml sample extract medium for 3 h and then challenged with 20 mM H_2O_2 for 1 h. Cells were removed by using trypsin/EDTA and washed twice with PBS. Cells were suspended in 0.25 ml PBS containing triton X-100 and sonicated in an ice water bath for 15 min to lyse the cells and then centrifuged at 10 000g, 4 °C for 5 min. The supernatant was collected to measure enzyme activity and the amount of protein was measured using BSA as standard at 595 nm[16].

Cytotoxicity assay

HepG2 cells were plated in 100 mm Petri dishes in groups of 2.5×10^6 cells. To study H_2O_2 cytotoxicity, 40 h after plating, the medium was discarded and fresh medium containing H_2O_2 at various concentrations was added. Cellular viability was determined by using trypan blue. In order to determine the concentration of plant extract that protects 50% of the cells from damage induced by the toxicant, cells were incubated with 20 mM of H_2O_2 for 2 h to induce significant cell death. Based on the dose-response curves of cell death protection by plant extract against the H_2O_2-induced oxidative damage in HepG2 cells, the IC_{50} concentrations were estimated and used in the following experiments to evaluate the protective potential of the compounds on several cellular functions.

Assay of glutathione S-transferase activity

Assays were performed using glutathione and 1-chloro-2,4-dinitrobenzene (CDNB)[17] in the presence of Triton X-100 (0.04%) at 30 °C, using an extinction coefficient of 9600 $M^{-1}cm^{-1}$ at 340 nm. Assays were performed at least in triplicate on each sample. Protein was assayed using the method of Bradford[16] using bovine serum albumin as a standard.

Lipid peroxidation

The extent of lipid peroxidation was estimated by the levels of malondialdehyde measured using the thiobarbituric acid reactive substances (TBARS) assay at 535 nm following a method previously described[18]. The results were expressed as nmol/mg of protein using a molar extinction coefficient of 1.56×10^5 $M^{-1}cm^{-1}$.

Total phenolic content

The assay was based on the method of Singleton and Rossi[19]. Briefly, 0.2 g of dried sample was extracted with 20 ml of a mixture of dimethylformamide and acetate buffer (pH 4.4) at room temperature for 16 h in the dark on a shaking water bath and filtered through Whatman paper in the hood. Each extract was diluted with deionized water. Aliquots of diluted filtrate (25 µl) were transferred to 96-well microplates and then mixed thoroughly with 125 µl of Folin-Ciocalteu reagent. After mixing for 1 min, 100 µl of 0.5 M sodium hydroxide was added. The mixtures were shaken briefly, and then allowed to stand for a further 15 min in the dark. The absorbance of plant extracts and a prepared blank were measured at 750 nm using a spectrophotometer. The concentration of total phenolic compounds in all plant extracts was expressed as mg of gallic acid equivalents (GAE) per 100 g freeze dried weight of plant.

DPPH assay

This was carried out and modified according to Fukumoto and Mazza[20]. DPPH is a free radical which is violet in colour. The antioxidants in the sample scavenge the free radicals and turn it yellow. The change of colour from violet to yellow is proportional to the radical scavenging activity (RSA). Diluted sample extracts (22 µl) were added to wells in a 96-well microtitre plate followed by 200 µl of 150 µM DPPH in 80% methanol. The plate was covered and left in the dark at room temperature for 30 min. The scavenging of free radicals by plant extracts was evaluated spectrophotometrically at 520 nm against the absorbance of the DPPH radical[20]. RSA was calculated as: (absorbance of blank−absorbance of extract)/absorbance of blank.

Percentage of RSA was plotted against the corresponding concentration of the extract to obtain IC_{50} values. IC_{50} is defined as the amount of antioxidant material required to scavenge 50% of free radicals in the assay system. The IC_{50} values are inversely proportional to the antioxidant activity.

Deoxyribose degradation assay

Hydroxyl RSA was measured by studying the competition between deoxyribose and test compounds for hydroxyl radical generated by the Fe^{3+}-ascorbate-EDTA-H_2O_2 system according to the method of Aruoma with slight modifications[21].

Superoxide dismutase (SOD) activity measurement

SOD activity was measured by the SOD assay kit-wst (19160 SOD determination kit; Fluka). Superoxide RSA study was determined by using the xanthine-xanthine oxidase system. Xanthine is converted to uric acid by the enzyme xanthine oxidase with the formation of a by-product, the superoxide. The superoxide combines with Nitro blue tetrazolium and forms formazine blue colour. The antioxidant-containing sample scavenges the superoxide, and thus the formation of formazine blue colour is reduced. The reduction of colour is proportional to the antioxidant content in the sample and the blue colour developed was measured at 450 nm.

Superoxide anion scavenging activity was calculated as $(E - S)/E$, where $E = A - B$, $S = C - (B + D)$, and A, B, C, and D are the optical densities of the control, control blank, sample, and sample blank, respectively.

Catalase activity measurement

CAT activity was determined by using a catalase assay kit (CAT 100, Sigma). The decomposition rate of the substrate H_2O_2 was monitored at 240 nm. A molar absorptivity of 43.6 $l\,mol^{-1}cm^{-1}$ was used to calculate the activity. One unit is equal to 1 µmol of H_2O_2 decomposition/min.

Statistic analysis

Each of the measurements described above was carried out in at least three replicate experiments. The normality of the data distribution was evaluated by the Kolmogorov-Smirnov test and the significant difference between groups at $p < 0.05$ was determined using a paired t-test.

RESULTS

Total phenolic content

The highest contents of total phenolics were found in fresh samples (Table 1). Boiling significantly decreased the total phenolic content ($p \leqslant 0.05$), but frying did not significantly decrease it ($p \geqslant 0.05$).

Table 1 Total phenolic content, IC_{50} values of DPPH scavenging, hydroxyl radical scavenging for non-processed and processed *C. hystrix* leaves.

Type of sample	Total phenolic content[a]	DPPH scavenging (mg/ml of extract)	Hydroxyl radical scavenging (mg/ml of extract)
Fresh	1940 ± 125^{B}	13.0	22.0
Boiled	1690 ± 55^{C}	11.9	35.8
Fried	1828 ± 81^{B}	19.9	44.0

[a] Data are means $\pm$ SD ($n = 4$) and expressed as mg GAE/100 g freeze-dried sample.
Different uppercase superscript letters indicate significant difference ($p \leqslant 0.05$).

DPPH radical scavenging activity

The IC_{50} value of the standard was found to be 0.5 mg/ml (Table 1). There was an inverse relationship between IC_{50} and antioxidant activity. The results indicated that fried samples possess the lowest DPPH scavenging activity among the samples. There was no effect of boiling samples on DPPH scavenging activity of *C. hystrix* leaves when compared with fresh samples. This result agreed with previous reports that scavenging activities towards DPPH of green leaves of potatoes blanched for 2 min at 100 °C remained the same as for fresh ones[22]. A linear relation between the total phenolic content and DPPH activities of the extract was not observed.

Hydroxyl radical scavenging activity

The interaction of iron ions with hydrogen peroxide in biological systems can lead to the formation of a highly reactive tissue-damaging species that is thought to be the hydroxyl radical[23,24]. The results indicated that fresh *C. hystrix* leaves possess the highest OH RSA (22 mg/ml) among the samples. Boiled samples had better OH RSA than the fried sample (Table 1). A linear relation between the total phenolic content and OH radical scavenging activity of the extract was not observed.

Inhibitory effects of *C. hystrix* leaf on lipid peroxidation induced by H_2O_2

Toxicity caused by ROS is normally accompanied by an increase of lipid peroxides[25]. In this study, the oxidative damage was evaluated by the formation of MDA. As indicated in Table 2, when HepG2 cells were exposed to H_2O_2 (20 mM) alone for 1 h, an increase in lipid peroxidation level, as indicated by the excessive formation of MDA in HepG2 cells, was observed to be twice that of the control values. However, co-incubation with fresh *C. hystrix* leaves inhibited the formation of MDA by 34%, while boiled and fried samples inhibited the production of MDA by 29% and 10%, respectively.

Glutathione-S-transferase activity

The incubation of HepG2 cells with 20 mM H_2O_2 for 1–2 h decreased cell viability by 40–60% along with a significant increase in lipid peroxidation. The toxicant also significantly decreased the GST activity. Pretreatment with *C. hystrix* leaf resulted in decreased GST activity (Table 2). This suggests that *C. hystrix* leaves have no chemopreventive potential.

Superoxide dismutase activity

This study showed that cooking had no effect on superoxide dismutase activities in HepG2 cells treated with *C. hystrix* leaves alone. Pretreatment with fresh, boiled, and fried *C. hystrix* leaves resulted in decreased superoxide dismutase activity (Table 2). This suggests that the presence of *C. hystrix* leaves does not result in enhanced scavenging of superoxide.

Catalase activity

The CAT activity in HepG2 treated with fresh and boiled *C. hystrix* leaves alone were twice as large as the control (Table 2). However, pretreatment with *C. hystrix* leaf extracts resulted in decreased CAT activities, which suggests that *C. hystrix* leaf extracts do not detoxify H_2O_2.

DISCUSSION

This study suggests that *C. hystrix* leaf may exert its antioxidative stress properties by scavenging hydroxyl radicals and inhibiting lipid peroxidation that causes oxidative damage to the liver cancer cell line HepG2. One of the possible components for inhibiting lipid peroxidation found in *C. hystrix* is myricetin. Myricetin is a predominant flavonol of *C. hystrix* leaves (68.4 mg/100 g fresh weight). It is present at a higher level (mg/100 g edible portion) than in other edible leaves such as Chinese kale (0.01), lettuce leaf (0.02), spinach (0.01), and sweet potato leaf (9.74)[26]. According to the study of Pandey et al, myricetin at micromolar concentration significantly protected a t-BHP-induced level of malondialdehyde (lipid peroxidation) of diabetic erythrocytes[27]. Moreover, the study of Hutadilok et al[28] also showed that the extract from leaves of *C. hystrix* exerted the strongest effect on production of the hydroxyl radical. It conferred a twice greater protection of deoxyribose from hydroxyl radicals than did tannin.

Table 2 Effect of *C. hystrix* leaves on lipid peroxidation, glutathione *S*-transferase (GST), superoxide dismutase (SOD) and catalase (CAT) induced by H_2O_2.

Sample	MDA (nmol/mg protein)		Reduction of MDA (-fold)		GST activity (nmol/min/mg protein)		SOD (units/mg protein)[a]		CAT (μmol of H_2O_2 consumed/min/mg protein)	
	(−)	(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)	(+)
Control	0.38 ± 0.04	0.77 ± 0.11	1	1	17.0 ± 0.7	10.9 ± 1.1	0.69 ± 0.13	1.01 ± 0.20^{B}	0.22 ± 0.03	0.33 ± 0.04
Fresh	0.36 ± 0.10	0.51 ± 0.12	1.05	1.51*	16.5 ± 0.5	9.1 ± 2.9	0.50 ± 0.05	0.75 ± 0.18^{BC}	$0.40 \pm 0.05^{\#}$	0.14 ± 0.01
Boiled	0.40 ± 0.08	0.55 ± 0.10	1.05	1.40*	12.7 ± 0.8	5.7 ± 0.6*	0.57 ± 0.03	0.61 ± 0.08^{C}	$0.31 \pm 0.06^{\#}$	0.03 ± 0.01
Fried	0.41 ± 0.05	0.69 ± 0.08	0.92	1.11	13.3 ± 3.7	7.3 ± 0.7	0.49 ± 0.10	0.50 ± 0.09^{C}	0.22 ± 0.86	0.03 ± 0.03

(−): without H_2O_2; (+): with H_2O_2.
The results represent the mean ± SEM of at least three separate experiments.
* Within a column $p \leqslant 0.05$ when compared with the H_2O_2 control.
[a] Within a column sharing the same superscripts are not significantly different from each other at $p \leqslant 0.05$.
Within a column $p \leqslant 0.05$ when compared with the negative control.

Hydrogen peroxide, in the presence of metal ions, is converted to a hydroxyl radical and a hydroxide ion. Pretreatment with *C. hystrix* leaf extracts resulted in decreased CAT activities, which suggests that *C. hystrix* leaf extracts do not directly detoxify H_2O_2. However it is possible that *C. hystrix* leaf extracts scavenge hydroxyl radicals.

Phenolics are the most widespread secondary metabolites in the plant kingdom. This diverse group of compounds has received much attention as potential natural antioxidants in terms of their abilities to act as both efficient radical scavengers and metal chelators[29]. Boiling had the most effect on *C. hystrix* polyphenolics, resulting in a loss of these substances in the boiling water. This is probably related to phenolic acids dissolved in vacuoles, and the apoplast determines softening and breaking of cellular components with the consequent release of these molecules into the boiling water in cooking of vegetables[30]. The increased softening property observed in our boiled sample explains the loss of phenolics in comparison with the fresh and fried sample[12]. Moreover, our study showed that the freeze-drying technique did not affect phenolic content in the sample.

CONCLUSIONS

This study suggests that *C. hystrix* leaf may exert its antioxidative stress by scavenging hydroxyl radical and inhibiting lipid peroxidation that causes oxidative damage to the liver cancer cell line HepG2. Boiling and frying decrease lipid peroxidation, GST activities, hydroxyl radical scavenging activity, and total phenolic contents in *C. hystrix* leaf. The data we report above, in conjunction with data reported by numerous other investigators, clearly indicate that dietary constituents can exert significant modulatory effects on cytotoxicity and oxidative reactions in cellular systems. Further studies are warranted to identify the active substance and principle present.

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