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Tamarind seed coat extract restores reactive oxygen species through attenuation of glutathione level and antioxidant enzyme expression in human skin fibroblasts in response to oxidative stress

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PEER REVIEW

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Comments

This work made an attempt to study on the local herbs collected from a country in Southeast Asia. The authors used standard method in ethnopharmacology to study the property of the collected herb. This work is a good example in ethnopharmacological studies and can be further applied for further usage in tropical medicine.
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ABSTRACT

Objective: To investigate the role and mechanism of tamarind seed coat extract (TSCE) on normal human skin fibroblast CCD-1064Sk cells under normal and oxidative stress conditions induced by hydrogen peroxide (H_2O_2).
Methods: Tamarind seed coats were extracted with boiling water and then partitioned with ethyl acetate before the cell analysis. Effect of TSCE on intracellular reactive oxygen species (ROS), glutathione (GSH) level, antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase activity including antioxidant protein expression was investigated.
Results: TSCE significantly attenuated intracellular ROS in the absence and presence of H_2O_2 by increasing GSH level. In the absence of H_2O_2, TSCE significantly enhanced SOD and catalase activity but did not affected on GPx. Meanwhile, TSCE significantly increased the protein expression of SOD and GPx in H_2O_2-treated cells.
Conclusions: TSCE exhibited antioxidant activities by scavenging ROS, attenuating GSH level that could protect human skin fibroblast cells from oxidative stress. Our results highlight the antioxidant mechanism of tamarind seed coat through an antioxidant enzyme system, the extract potentially benefits for health food and cosmeceutical application of tamarind seed coat.

KEYWORDS

Tamarindus indica, Seed coat, Antioxidant, Reactive oxygen species, Glutathione, Antioxidant enzymes, CCD-1064Sk cells

1. Introduction

Oxidative stress in living cells involves the production of reactive oxygen species (ROS), such as superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$) and hydrogen peroxide (H_2O_2). ROS can cause lipid peroxidation, protein denaturation and oxidative damage of DNA that resulting in many diseases[1]. After exposure to oxidative stress, the defense mechanisms including an enzymatic system such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and non-enzymatic system such as glutathione (GSH), β-carotene (vitamin A), ascorbic acid (vitamin C) and α-tocopherol (vitamin E) are involved[1,2]. Dietary antioxidants such as vitamin A, vitamin C and vitamin E including plant polyphenolic compounds play health benefits better and more safely than synthetic

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antioxidants such as butylated hydroxytoluene and butylated hydroxyanisole[3]. Natural antioxidants possess scavenging activity against various free radicals[4,5], inhibiting lipid peroxidation[5] and attenuating intracellular ROS through antioxidant enzymes activity[6]. In a skin, free radicals induced by UV radiation can cause damage to DNA, protein, and fatty acid which leads to skin photoaging and photocarcinogenesis[7]. Phytoantioxidants, such as phenolic acids, flavoniods and high molecular weight polyphenols can protect the skin from UV radiation which can be applied for skin care formulation[7].

Tamarindus indica L. (in family Fabaceae and subfamily Caesalpiniodeae) commonly known as tamarind, is originally native tree to Africa and is now worldwide cultivated in many tropical countries. Tamarind is widely used in various traditional medicine and food products[8]. The fruits can be consumed fresh and as an ingredient in many kinds of Thai food[9]. Tamarind seed, a by-product from food industries, have been used as a substitute for coffee for a long time[10]. The seed consists of a kernel and seed coat. The seed coat is a rich source of tannins and polyphenols which possesses antiallergic and antimicrobial[11], antibiotic[12], antityrosinase[13], and antioxidant activities[14,15]. However, non-enzymatic and enzymatic antioxidant activities of tamarind seed coat at cellular level are still unexplored. The present study aimed to investigate whether tamarind seed coat extract (TSCE) could protect against H_2O_2-induced oxidative stress in human foreskin fibroblast CCD-1064Sk cells through antioxidant defense mechanism. The cells have been widely used as a model to evaluate wound healing processes[16], and anti-skin aging[17].

2. Materials and methods

2.1. Plant material and preparation of tamarind seed coat extract

Ripened tamarind pods of the sour tamarind, *Tamarindus indica* "Priao-Yak", were collected from Phetchabun, Thailand. Herbarium specimens were deposited at the Museum of Natural Medicine, Faculty of Pharmaceutical Sciences, Chulalongkorn University. The tamarind seeds were separated and roasted in a pre-acid washed sand-bath at 150 °C for 1 h. The seeds were then quickly washed and dried in a hot air oven at 50 °C for 24 h. The seed coats were removed from the kernels, pulverized into a tamarind seed coat powder (TSCP). TSCP was extracted as previously described[18], with slightly modified. Three grams of TSCP was put in a cloth sack, dipped while shaking in 200 mL boiling-water and boiled for 2 min. The sack was pressed and the red water extract was collected. The residue was then re-extracted until the extract was colorless. The extract was pooled and filtered. Before the cell analysis, the clarified extract was further partitioned with an equal volume of ethyl acetate and then concentrated by rotary evaporator (Buchi, Switzerland) at 40 °C and dried under blowing nitrogen gas. The dried TSCE was dissolved in dimethyl sulfoxide (DMSO) at 400 mg/mL stock solution and serial dilutions were prepared in the culture medium before used.

2.2. Cell culture

CCD-1064Sk cells were purchased from American Type Culture Collection (CRL-2076, VA, USA). The cells were grown and maintained in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum, 100 IU/mL penicillin and 100 μg/mL streptomycin and maintained at 37 °C in humidified 5% CO_2 incubator.

2.3. Determination of cell viability

Cell viability was determined using neutral red assay[19], with slightly modified. The cells were seeded in 96-well plates at a density of 1×10^5 cells/mL and incubated for 24 h. After removal of medium, the cells were treated with 100 μL of TSCE at concentrations ranging from 0.05 to 1 mg/mL for 24 h. The TSCE was removed and then washed twice with 100 μL of phosphate buffer solution (PBS) (pH 7.4) and then H_2O_2 was added to induce the cell damage. About 100 μL of 40 μg/mL neutral red medium was added and incubated for 3 h. After incubated the neutral red medium was removed and rapidly washed and fixed with 100 μL of fixing solution. The dye was extracted by adding 100 μL of extraction solution, incubated for 20 min, and then agitated on a rocking platform for 5 min. An absorbance was measured at 570 nm using micropalte reader (Wallac 1420, Perkin Elmer, USA). The cell viability was calculated compare with vehicle control (0.25% DMSO in medium).

2.4. Determination of intracellular ROS

The intracellular ROS level was investigated using dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay[20]. Cells were seeded into 96-well plates at a density of 1×10^5 cells/mL and incubated for 24 h. The volume 100 μL of TSCE at concentrations ranging from 0.05 to 1 mg/mL was added and incubated for 24 h and then washed twice with cold PBS. Thereafter, 100 μL of 5 μmol/L DCFH-DA was added and incubated for 30 min. The cells were washed and then 1 mmol/L H_2O_2 was added and incubated for 15 min for generating intracellular ROS. The fluorescence intensity of DCF was measured by using a microplate reader at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

2.5. Determination of intracellular GSH level

Total level of intracellular GSH was measured by using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB)-glutathione disulfide (GSSG) reductase recycling assay[21]. Cells were seeded into 6-well plates at a density of 1×10^5 cells/mL and incubated for 24 h. Thereafter, the cells were treated with TSCE at concentrations ranging from 0.05 to 1 mg/mL for 24 h and then exposed with 1 mmol/L H_2O_2 for 30 min. The cells were lysed, deproteinized, and centrifuged. The supernatant of cell lysates were determined. The reaction mixture contained 10 μL of cell lysates, 90 μL of 0.1 mol/L sodium phosphate buffer containing 1 mmol/L ethylene diamine tetraacetic acid (pH 7.5), 10 μL of 10 IU/mL glutathione reductase, 80 μL of 0.25 mmol/L NADPH and 10 μL of 1.5 mmol/L DTNB, respectively. The rate of TNB production

was measured at 405 nm by using a microplate reader at 30 seconds intervals for 10 min. The total intracellular GSH level was analyzed by the kinetic method from a linear standard curve of GSH. The value was expressed in μmol/L per mg protein.

2.6. Determination of antioxidant enzymes activity

The cells were seeded into 6-well plates at a density of 1×10^5 cells/mL and incubated for 24 h. After removal of medium, the cells were treated with TSCE at concentrations ranging from 0.05 to 1 mg/mL for 24 h and then exposed to H_2O_2. The cells were washed twice with cold PBS, trypsinized and centrifuged at 1 040 r/min (by using EBA 12, Hettich Zentrifugen, Tuttlingen, Germany) at 4 °C for 5 min. Cell pellets were washed twice with cold PBS and then SOD, GPx and CAT activity were determined.

2.6.1. Determination of SOD activity

The cell pellets were re-suspended in 600 μL of 50 mmol/L $NaHCO_3$ buffer (pH 10.2). Thereafter, the cells were lysed on ice by using ultrasonic disintegrator at 5 μm of amplitude every 2 seconds for 10 seconds. Cell lysates were centrifuged at 12 500 r/min at 4 °C for 10 min. The supernatant of cell lysate was determined for SOD activity[22].

2.6.2. Determination of GPx and CAT activity

The cell pellets were re-suspended in ice-cold 0.013% sodium cholate (20×10^6 cells/mL), sonicated on ice every 2 seconds for 10 seconds and then centrifuged at 9 660 r/min at 4°C for 10 min. The supernatant of cell lysate was determined for GPx and CAT activity[23].

2.7. Western blot analysis

The cells were seeded at a density of 1×10^5 cells/mL into 6-well plates and incubated for 24 h. After removal of medium, the cells were treated with TSCE at concentrations ranging from 0.4 to 0.8 mg/mL for 24 h. Thereafter, the cells were exposed with 1 mmol/L H_2O_2 for 3 h. After removal of medium, the cells were washed twice with ice-cold PBS, scraped, collected, and centrifuged at 1 500 r/min at 4°C for 8 min. The protein was extracted by radioimmunoprecipitation assay lysis buffer on ice for 30 min. After centrifuged at 12 000 r/min at 4°C for 10 min, the supernatant was collected and protein concentration was measured. The protein extract was mixed with loading buffer, boiled at 95 °C for 5 min. The amount of 30 mg proteins of each sample were loaded onto 12% sodium dodecyl sulfate-polyacrylamide gel and electrophoresis at 90 V for 1.5 h. The gel was further transferred onto polyvinylidene fluoride membrane by electroblotting at 45 V for 2 h. The membrane was incubated with 5% bovine serum albumin in tris buffered saline Tween buffer for 2 h at room temperature for blocking non-specific binding protein. Blots were probed with primary rabbit antibodies (Cu,Zn-SOD or GPx or CAT from Abcam, Cambridge, England) at 4 °C overnight. The membrane was washed 6 times with tris buffered saline Tween and then blotted with a goat anti-rabbit polyclonal secondary antibody with horseradish peroxidase conjugated for 1 h at room temperature. The protein bands were detected by enhanced chemiluminascence detection reagent and exposed to Kodak X-ray films. The intensities of the bands were visualized and computed by ImageJ 1.43u software (NIH, Bethesda, MD, USA).

2.8. Statistical analysis

The SPSS program was used for statistical analysis. Data were expressed as mean±SEM obtained from each test in triplicate. Statistical analysis was performed using One-way analysis of variance (ANOVA), followed by Turkey's *post hoc* mean comparison. A level of $P<0.05$ was considered statistically significant.

3. Results

3.1. Effect of TSCE on intracellular ROS

Effect of the TSCE polyphenols on intracellular ROS level as compared with viability was determined using DCFH-DA and neutral red assays, respectively. The result demonstrated that TSCE alone decreased intracellular ROS in a concentration-dependent manner (Figure 1A). To optimize oxidative stress condition, the cells were treated with

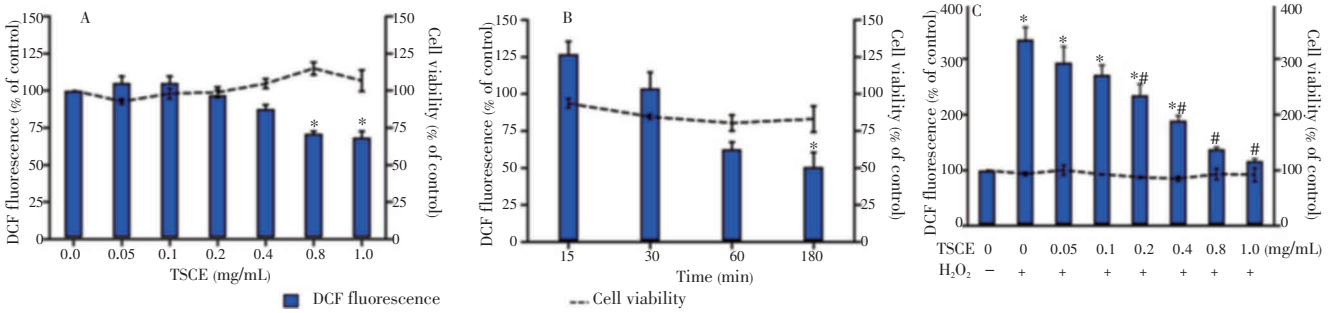

Figure 1. Effect of TSCE on the intracellular ROS generation and viability.

The cells were treated with (A) TSCE (0.05-1 mg/mL) for 24 h or (B) H_2O_2 (1 mmol/L) for 15, 30, 60 and 180 min. For oxidative stress condition, (C) treatment of cells with TSCE (0.05-1 mg/mL) for 24 h was further preloaded with DCFH-DA (5 μmol/L) for 30 min followed by exposure to H_2O_2 (1 mmol/L) for 15 min. The intracellular ROS level was measured as compared to DCF fluorescence intensity. The cell viability was determined using neutral red assay. Data are expressed as mean±SEM of three independent experiments. Each performed in triplicate. $^*P<0.05$ compared to vehicle control, and $^\#P<0.05$ compared to H_2O_2-treated cells without TSCE.

H_2O_2 at various concentrations and time points. The result demonstrated that an exposure with 1 mmol/L H_2O_2 for 15 min induced the highest oxidative stress (126.97%) without affecting cell viability (93.72%) (Figure 1B). Meanwhile, TSCE (0.2 to 1 mg/mL) significantly attenuated ROS in H_2O_2-treated cells (Figure 1C).

3.2. Effect of TSCE on intracellular GSH level

The effect of TSCE in the absence and presence of H_2O_2 on intracellular GSH was investigated using DTNB-GSSG reductase recycling assay. Treatment of the cells with H_2O_2 (1 mmol/L) for 30 min significantly decreased GSH level with low effect on cell viability (84.56%). The result showed that TSCE significantly increased total GSH level with the absence or presence of H_2O_2 in a concentration-dependent manner (Figure 2). Noticeably, the potency of TSCE in H_2O_2-treated cells was 2-fold stronger than TSCE treated alone.

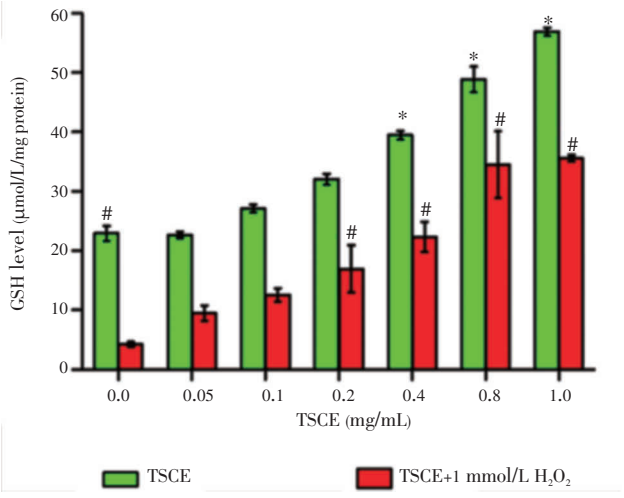

Figure 2. Effect of TSCE on total GSH level.

The cells were pretreated with TSCE (0.05-1 mg/mL) for 24 h and then exposed to H_2O_2 (1 mmol/L) for 30 min. Total GSH level was determined using DTNB-GSSG reductase recycling assay. Data are expressed as mean±SEM of three independent experiments. $^*P<0.05$ compared to control, and $^\#P<0.05$ compared to H_2O_2-treated cells.

3.3. Effect of TSCE on antioxidant enzyme activity

The role of enzymatic antioxidant system was further investigated. The result showed that TSCE treated alone at the concentration to decrease intracellular ROS significantly elevated SOD and CAT activities (Figure 3A). However, TSCE did not increase the activities of SOD, GPx and CAT in H_2O_2-treated cells (Figure 3B).

3.4. Effect of TSCE on protein expression of antioxidant enzymes

To evaluate the role of antioxidant enzyme expression, Western blot analysis was performed. The result showed that TSCE, at the concentrations with significant increase in SOD and CAT activities (Figure 3A), did not alter the protein expression of Cu,Zn-SOD, GPx including CAT (Figure 4). However, TSCE in H_2O_2-treated cells significantly up-regulated the protein expression of Cu,Zn-SOD and GPx but not for CAT as compared to the vehicle control (Figure 5).

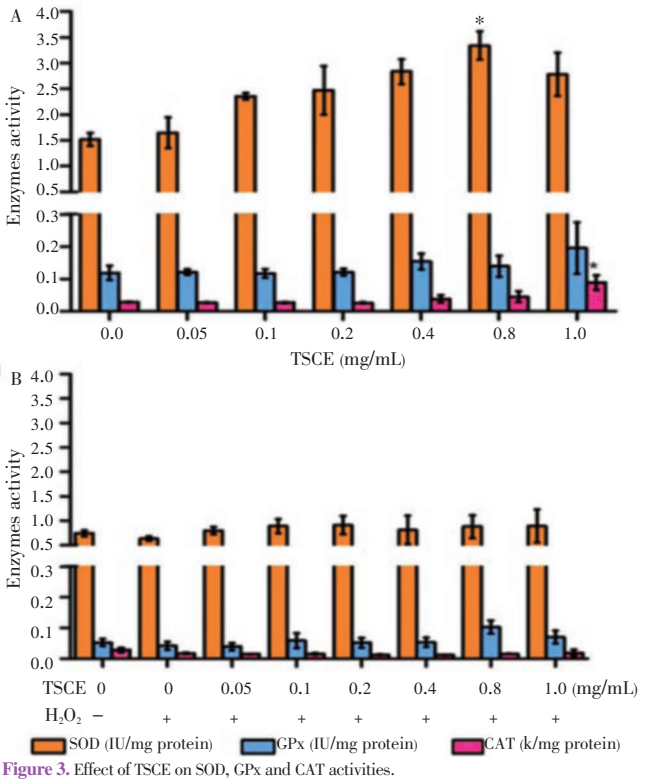

Figure 3. Effect of TSCE on SOD, GPx and CAT activities.

The cells were treated with TSCE (0.05-1 mg/mL) for 24 h either TSCE alone (A) or in the presence of H_2O_2 (2 mmol/L) for 15 min for SOD and 30 min for GPx and CAT (B). The activities of SOD, GPx and CAT were determined. Data are expressed as mean±SEM of three independent experiments. $^*P<0.05$ compared to vehicle control.

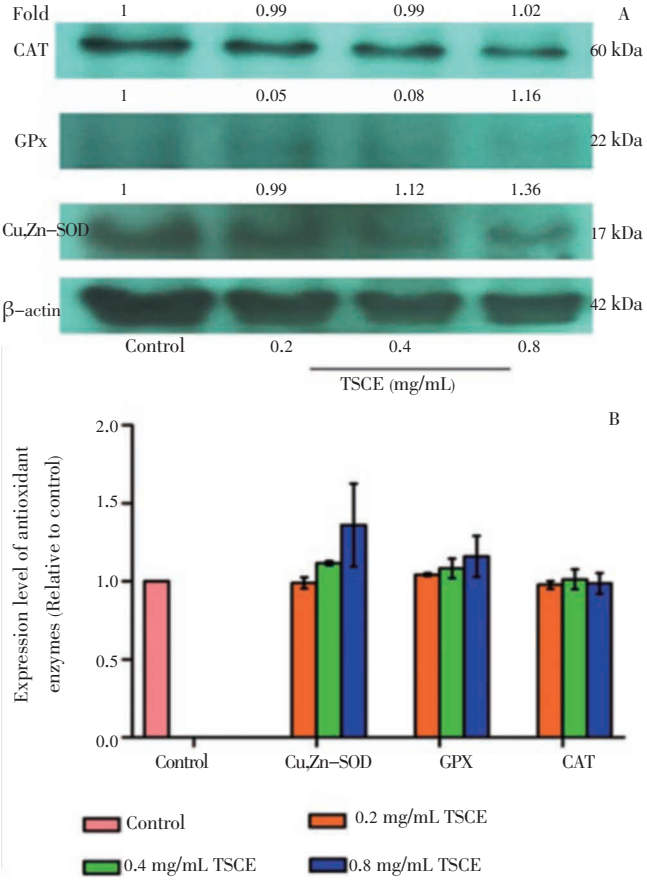

Figure 4. Effect of TSCE on protein expression of Cu,Zn-SOD, GPx and CAT.

The cells were treated with TSCE (0.2-0.8 mg/mL) for 24 h. The cell lysate was subjected to electrophoresis on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis with subsequent enzyme immunoblot assay. (A) Representative immunoblot and (B) corresponding cumulative data. The intensity of each band was normalized using β-actin. Data are expressed as mean±SEM of three independent experiments.

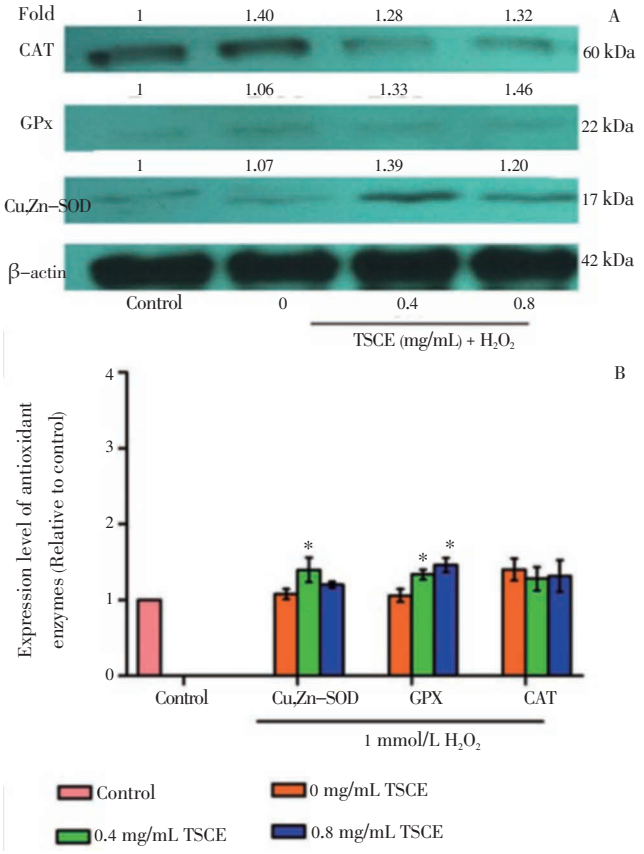

Figure 5. Effect of TSCE on protein expression of Cu,Zn-SOD, GPx, and CAT in H_2O_2-treated cells.

The cells were treated with TSCE at 0.4-0.8 mg/mL for 24 h followed by 1 mmol/L H_2O_2 for 3 h. (A) Cu,Zn-SOD, GPx, and CAT protein levels and (B) the intensity of each band after incubated with 1 mmol/L H_2O_2 for 3 h. Data are expressed as mean±SEM (n=3). $^*P$<0.05 compared to the vehicle control.

4. Discussion

It is well known that oxidative stress play an important role in aging and associated various diseases[1,7]. The scavenging ability of ROS, cytoprotective effects, antioxidant enzyme activity, and antioxidant mechanism of natural antioxidants are widely investigated[6,24,25]. The present study contributed to our preliminary report showed the underlying antioxidant defense mechanism of tamarind seed coat in CCD-1064Sk cells under oxidative stress. Our unpublished data also found that TSCE composed of phenolic compounds such as (+)-catechin, (-)-epicatechin and procyanidin B2, which is similar to the previous reports[13,26], with its concentrations ranging from 0.002 to 1 mg/mL, and it also possessed various antioxidant activities in cell-free system without affecting cell viability. We therefore determined that whether TSCE at the nontoxic concentrations could attenuate intracellular ROS level via an elevation of the non-enzymatic and enzymatic antioxidant system in the absence and presence of oxidative stress. H_2O_2, a precursor of ROS, was commonly applied as an oxidant reagent in the studies of various cell lines[24,25,27]. Therefore, H_2O_2 was used to induce cellular stress in this study.

Our result found that TSCE possessed activity to scavenge ROS under normal and oxidative stress conditions in CCD-1064Sk cells, it may be related to the $O_2^{\bullet-}$, $OH^{\bullet}$, and H_2O_2 scavenging ability of tamarind seed coat in cell-free system which was observed in our laboratory as well as in other published documents[15,26]. The potential ability of TSCE to scavenge ROS was due to an evaluation of GSH, which is a major intracellular non-enzymatic antioxidant in the detoxification of varieties of electrophilic compounds and peroxide via catalysis by glutathione S-transferase and GPx[28]. This result indicated that tamarind seed coat may be able to elevate GSH level in CCD-1064Sk cells as same as the study in the bark of tamarind[29].

Moreover, our result also demonstrated that TSCE elevated SOD and CAT activities but did not affect on GPx activity. Considering antioxidant enzymes activity in oxidative stress condition, H_2O_2 at the IC_{50} value as our study was also used to study the decrease of SOD, GPx and CAT activities in various cell lines such as osteoblast[27], Chinese hamster lung fibroblast (V79-4)[30] and human dermal fibroblast[31]. We therefore selected H_2O_2 at the concentration of 2 mmol/L (IC_{50}) in our testing model. The result showed that TSCE did not increase SOD, GPx and CAT activities due to the presence of high level of H_2O_2. Our finding was agreed with the previous reports, demonstrating that SOD played a major role to scavenge superoxide anion but did not scavenge H_2O_2[2]. Surprisingly, our study revealed that TSCE increased GSH level, one of GPx substrates, but did not increase GPx activity. This result revealed that the activity of GPx is independent on GSH level in case of initial concentration of GPx is higher than initial concentration of H_2O_2[32]. This finding was corresponding with some documents reported that the elevation of GSH did not affect on GPx activity[33,34] while it may affect on glutathione reductase or glutathione S-transferase[33]. Moreover, various concentrations of H_2O_2 effect on antioxidant enzymes responded differently[35]. Therefore, to gain insight into the mechanism of these enzyme systems, the effect of TSCE on antioxidant enzymes activity in oxidative stress induced by various concentration of H_2O_2 will be further studied.

TSCE did not increase the antioxidant enzyme expression in normal condition. However, TSCE could up-regulate the protein expression of Cu,Zn-SOD and GPx under oxidative stress at sub-concentration of H_2O_2. This finding was corresponding with the previous reports, revealing that the expression of anti-oxidative or protective genes were observed[36,37].

In summary, this study clearly demonstrated that TSCE exhibited antioxidant activity by increasing GSH level in

CCD-1064Sk cells under normal and mild oxidative stress condition induced by H_2O_2. Tamarind seed coat activated the antioxidant enzyme activity of SOD and CAT under normal cell condition as well as enhanced the protein expression of SOD and GPx under mild oxidative stress. Tamarind seed coat phenolic compounds can be simply extracted with boiling water, the extract may be used as a natural source of antioxidant and protective agent that may be useful as health promoting food and cosmeceutical application for skin aging.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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Comments

Background

Tamarindus indica L. is originally native tree to Africa and widely used in various traditional medicine and food products. The seed of this plant consists of a kernel and seed coat. The seed coat is a rich source of tannins and polyphenols which possesses antiallergic and antimicrobial, antibiotic, antityrosinase, and antioxidant activities. However, non-enzymatic and enzymatic antioxidant activities of tamarind seed coat at cellular level are still unexplored

Research frontiers

A new information on a local herb study center from a developing tropical countries. The information is interesting and can be further reference in ethnopharmacology.

Related reports

There are some previous reports on ethnopharmacology studies from Thailand. However, those previous ones did not focus on the present studied herbs. Hence, the work can be a good original data for followers.

Innovations and breakthroughs

The study is a proof of the property of ethnopharmaceuticals and it can be further developed for new drugs in the field that can be useful for the local and regional medicine.

Applications

As note, the result can be further references in herbal medicine and drug development. This kind of study is not widely available. The application can be warranted.

Peer review

This work described an attempt to study on the local herbs collected from a country in Southeast Asia. The authors used standard method in ethnopharmacology to study the property of the collected herb. This work is a good example in ethnopharmacological studies and can be further applied for further usage in tropical medicine.

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