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The effect of Indian marsh fleabane (*Pluchea indica* (L.) Less) dried leaves extract against oxidative stress induced by hydrogen peroxide in *Saccharomyces cerevisiae*

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Abstract

The objective of this research was to assess the potential antioxidative capacity of Indian marsh fleabane (*Pluchea indica* (L.) Less) dried leaves extract. The extract was investigated the content of major groups of antioxidants, radical scavenging capacity and the effect against oxidative stress which was induced by hydrogen peroxide in *Saccharomyces cerevisiae*. The results showed that the extract had high concentration of antioxidants; total phenolic compounds (84.69±1.56 mg GAE/g dry weight), total tannins (69.63±0.39 mg TAE/g dry weight) and total flavonoids (45.15±1.08 mg QUE/g dry weight). The extract showed high DPPH radical scavenging activity (IC_{50} = 0.04±0.00 mg/ml extract and TEAC = 81.04±0.55 mg TE/g dry weight). Cellular antioxidative stress activity of the extract was determined against the stationary phase *S. cerevisiae* TISTR 5240 which was induced to be stressed by hydrogen peroxide (H_2O_2) at IC_{50} concentration (1.88 mM). The results revealed that the highest survival rate of *S. cerevisiae* was at 100 μg/ml of the extract (%survival = 60.74±1.12) or 3/4 fold with respected to that of 50 μg/ml standard quercetin (%survival = 83.11±0.89). Survival rate of *S. cerevisiae* at the concentrations between 5.00–25.00 μg/ml were significantly lower (p<0.05) compared with the control, while that of 50.00 μg/ml of the extract and the control were not significant difference (p>0.05). These concentrations might be too low to scavenge free radicals. However, survival of yeast cells slightly increased with increasing the concentration of the extract pretreated to the cells. Importantly, survival rate of *S. cerevisiae* was significantly decreased when they were incubated with 500 and 1,000.00 μg/ml of the extract due to the toxicity of the extract to the cell. From the results, *P. indica* dried leaves extract had high antioxidant capacity and showed antioxidant activity against H_2O_2 at the cellular level or cytoprotective effects. Thus, dried leaves from *P. indica* may be developed to the herbal tea recommended to the consumers for potential preventing the diseases caused by oxidative stress.

Keywords: Indian marsh fleabane (*Pluchea indica* (L.) Less), cellular antioxidative stress, *Saccharomyces cerevisiae*

1. Introduction

Reactive oxygen species (ROS), such as ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\cdot}$), are by-products from aerobic metabolism of most cells. Naturally, the antioxidant enzymes protect cells from ROS, for example; superoxide dismutase and peroxidase [1]. When the processes are not balanced, oxidative stress will occur and will cause the cellular aging, cancer, cardiovascular diseases, immune system decline and other pathophysiological modifications [2]. So, it should be effort to find the effective plant-derieved substances with antioxidant property and antioxidative stress in cells.

Indian marsh fleabane (*Pluchea indica* (L.) Less.) is the shrub plant that belongs to the family of Asteraceae (Compositae). It is commonly found in mangrove and littoral areas of Southeast Asia, particulary in Thailand.

Its simple oval-like and margin tooths leaves are 1.0–5.0 cm width and 2.5–10.0 cm length. Previous studies noted that *P. indica* had the therapeutic potentials, for instances; diuretic treatment, anti-ulcer, anti-diabetic, anti-pyretic, antimicrobial and anti-inflammatory [3,4]. There was plenty of many antioxidants found in *P. indica*; such as polyphenolics, flavonoids, quercetin, quinic acid, tannins, lignin glycoside, terpenes and triterpenoids. It also showed lipid peroxidation inhibition, *Entamoeba Histolytica* HM1 inhibition, lipopolysaccharide-induced nitric oxide and prostaglandin E2 production inhibition [5-9]. Moreover, antioxidant and antibacterial activities of *P. indica* fresh sample were higher than that of dry samples, especially in fresh root extract. However, dried leaves extract had high content of phenolic compounds and DPPH radical scavenging capacity [10]. It might be due to the formation of Maillard reaction products during heating promoting the high antioxidative power.

In the past years, *P. indica* leaves has been consumed fresh or dry as food and natural medicine for preventing many diseases. Nowadays, *P. indica* herbal tea is more widely consumed as an alternative healthy drink. Nevertheless, many studies showed the antioxidant activity of *P. indica* leaves extract using only chemical methods; such as DPPH (2,2-diphenyl-1-picryl-hydrazyl), ATBS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) and FRAP (Ferric reducing antioxidant power) assays. However, there were no study about the antioxidative activity of them at a cellular level. Generally, a short life-cycle and easy to grow eukaryotic cell such as yeast *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* were preferred in the study of cellular antioxidative stress [1,11-13]. Yeast cells were incubated with the extract, then treated with oxidative stress-induced substance, and survival rate of them were investigated at the end of the experiment. The extract with high survival rate indicated that it had high antioxidatve stress capacity. Thus, the objectives of this research were to assess the effect of *P. indica* dried leaves extract against oxidative stress which was induced by hydrogen peroxide in *S. cerevisiae*; and to investigate the content of major groups of antioxidants and radical scavenging capacity. The results of this research might be useful as supporting data to develop the herbal tea from *P. indica* in the future.

2. Materials and Methods

2.1 Chemicals

All chemicals used in the study were analytical reagent grade: Gallic acid (Sigma-Aldrich USA), DPPH (2,2-diphenyl-1-picryl-hydrazyl, Sigma-Aldrich USA), Trolox (Sigma-Aldrich USA), Folin-Ciocalteu phenol reagent (Loba Chemie India), Sodium carbonate (Na_2CO_3, Univar Ajax Finechem New Zealand), Methanol (Merck Germany), Tannic acid (Alfa Aesar UK), Quercetin (Sigma–Aldrich USA), Aluminium chloride ($AlCl_3$, Univar Ajax Finechem New Zealand), Potassium acetate (CH_3COOK; Unilab Australia), Hydrogen peroxide (H_2O_2; Fisher Scientific UK), Potato Dextrose Agar (PDA, Himedia India), Disodium hydrogen phosphate (Na_2HPO_4; Univar Ajax Finechem Australia), Sodium dihydrogen phosphate (NaH_2PO_4; Univar Ajax Finechem Australia) and Sodium chloride (NaCl; Univar Ajax Finechem Australia).

2.2 Plant materials

Pluchea indica (L.) Less leaves samples were manually picked from surrounding areas of Chanthaboon Handicraft Center in Tambon Bang-Sa-Kao, Laem Sing District, Chanthaburi Province, Thailand in January 2016. Samples were identified by the Department of Plant Production Technology and Landscape, Faculty of Agro-Industrial Technology, Rajamangala University of Technology Tawan-Ok, Chanthaburi Campus. After that, fresh leaves samples were washed with water, blanched with boiling water for 5 min, then spreaded out for withering. After that, leaves were processed into dried leaves by pan firing at 50 ^oC for 2 h, and then dried in hot air oven at 50 ^oC for 2 h to avoid moisture and mold. Dried leaves were stored in a plastic-zip lock bags and kept in a desiccator at room temperature before analysis.

2.3 Preparation of yeast cells

Saccharomyces cerevisiae TISTR 5240 was derived from Thailand Institute of Scientific and Technological Research (TISTR Culture Collection). Yeast cells were grown in Potato Dextrose Agar (PDA) medium to reach the stationary phase, followed by centrifugation at 3,000 rpm for 3 min. After that, yeast cells were collected, washed in triplicated, and diluted with Phosphate Buffer Saline (PBS) (0.1 M phosphate buffer pH 6.9 containing 6.7 mM NaCl) to yield $2x10^8$ CFU/ml. Finally, yeast suspension was incubated at 30 ^oC, 160 rpm for 1 h before use.

2.4 Extraction

Dried leaves samples were macerated using 70% ethanol at a ratio of 1:5 w/v overnight and the residues were re-macerated with the same condition. The extracts were combined, filtered and evaporated using rotary-evaporator at 70 ^{o}C to yield the crude extract.

2.5 Chemical assays for antioxidants and antioxidant activity

2.5.1 Total phenolics content

Total phenolics content was measured using Folin-Ciocalteu method [14] with slightly modifications. An aliquot of 2.0 ml of the extract (dried extract in 1:1 v/v of methanol: water) was incubated with 5.0 ml of 10% Folin-Ciocalteu phenol reagent, and followed by adding of 2.0 ml of 7.5% Na_2CO_3. The mixture was shaken and left in the dark at room temperature for 1 h, then the absorbance was measured at 765 nm. Total phenolics content was calculated from the standard curve of gallic acid. The result was expressed as milligram gallic acid per gram of the extract (mg GAE/g dry weight).

2.5.2 Total tannins content

Total tannins content was estimated using Folin-Ciocalteu method [15] with a little modifications. An aliquot of 2.0 ml of the extract (prepared as described above) was mixed with 1.0 ml of 10% Folin-Ciocalteu phenol reagent, then it was added with 2.0 ml of saturated Na_2CO_3 and 5.0 ml of double-distilled water. After 1 h, the mixture was measured at 725 nm. Total tannins content was calculated from the standard curve of tannic acid. The result was expressed as milligram tannic acid per gram of the extract (mg TAE/g dry weight).

2.5.3 Total flavonoids content

Total flavonoids content was determined using Aluminium chloride method [16]. An aliquot of 0.3 ml of the extract was made up to 2.0 ml with methanol. Then, 0.1 ml of 10% $AlCl_3$ was added, followed by 0.1 ml of 1 M CH_3COOK and 2.8 ml of double-distilled water. The absorbance was recorded at 415 nm after 30 min at room temperature. Total flavonoids content was calculated from the standard curve of quercetin and the result was expressed as milligram quercetin per gram of the extract (mg QUE/g dry weight).

2.5.4 DPPH radical scavenging activity

Antioxidant activity was determined using DPPH assay [17]. Briefly, an aliquot of different concentrations of the extract was mixed with 4.5 ml of 0.1 mM DPPH and then diluted to 5.0 ml with double-distilled water. After the mixture was left for 30 min, the absorbance was measured at 515 nm. Antioxidant activity were reported as the concentration of antioxidants that inhibited or scavenged 50% of 0.1 mM DPPH or IC_{50} (mg/ml extract) and Trolox equivalent antioxidant capacity or TEAC (mg TE/g dry weight).

2.6 Cellular antioxidative stress assay

2.6.1 IC_{50} of hydrogen peroxide

Determination of IC_{50} of hydrogen peroxide was performed using the method modified from Wiyakrutta et al. [13]. The yeast suspension was made up to yield $1x10^4$ CFU/ml with PBS and initial cells viability was measured using Dilution spread plate method in PDA. An aliquot of 100 µl of yeast suspension was dropped into microcentrifuge tubes, further exposed to H_2O_2 which the final concentrations were 0.50, 1.00, 2.00 and 3.00 mM, and brought up to 200 µl with sterile double-distilled water. Control was also conducted with sterile double-distilled water. The experimental culture was incubated at 30 ^{o}C, 260 rpm for 1 h. The viable yeast colonies after the exposure were counted using Dilution spread plate method in PDA. The percentage of yeast survivals were plotted against the concentration of H_2O_2 to find out the concentration of H_2O_2 that killed 50% of yeast cells due to the oxidative stress or IC_{50}.

2.6.2 Antioxidative stress assay

Antioxidative stress activity at a cellular level of *P. indica* dried leaves extract was performed using the method as described by Wiyakrutta et al. [13]. The 50 µl of yeast suspension in PBS ($2x10^8$ CFU/ml); which was incubated at 30 ^{o}C, 160 rpm for 1 h from the preparation step; was mixed with an aliquot of 950 µl of

various concentrations of *P. indica* dried leaves extract (5.00, 10.00, 25.00, 50.00, 100.00, 500.00 and 1,000.00 μg/ml). Standard quercetin (50.00 μg/ml in methanol) and sterile double-distilled water were used as the standard and the control, respectively. The cell viability at the beginning of each treatment was counted using Dilution spread plate method in PDA. The 200 μl of each treatment was dropped into microcentrifuge tube and incubated at 30 ºC, 260 rpm for 1 h. Then, the mixture was challenged to 200 μl of H_2O_2 at IC_{50} concentration and further re-incubated at 30 ºC, 260 rpm for 1 h. The viable yeast colonies after the incubation were counted using Dilution spread plate method in PDA to evaluate the percentage of survival against the initial viability.

2.7 Statistical analysis

All experiments were investigated in triplicate and the data were expressed as mean ± standard deviation. The data were analyzed using one-way analysis of variance (ANOVA). The differences among samples were determined by t- test at a level of $p<0.05$ of significance.

3. Results and Discussions

3.1 Chemical assays for antioxidants and antioxidant activity

Extraction of *P. indica* dried leaves using 70% ethanol (1:5 w/v) yielded 40.47 mg/g dry weight. Concentration of major groups of antioxidants and antioxidant activity are shown in Table 1. It revealed that *P. indica* dried leaves extract had high concentration of antioxidants; total phenolic compounds (84.69±1.56 mg GAE/g dry weight), total tannins (69.63±0.39 mg TAE/g dry weight) and total flavonoids (45.15±1.08 mg QUE/g dry weight). These compounds contributed the antioxidant activity of *P. indica* [4,5,18,19].

Because hydrogen peroxide was used to induced the oxidative stress in this research, DPPH assay was practiced to evaluate the free radicals scavenging properties. From the results, the extract showed a high DPPH radical scavenging activity (IC_{50} = 0.04±0.00 mg/ml extract and TEAC = 81.04±0.55 mg TE/g dry weight). When the results were compared with the previous study, the antioxidant activity in this study was higher than that of hot water extract of *P. indica* dried leaves (EC_{50} = 0.02±0.00 mg/ml) [8]. It might be due to the difference in solvent and extraction conditions. However, chemical assays could demonstrate only primarily potential of antioxidant capacity in the extract. Thus, the cellular antioxidative stress activity were approached.

Table 1 Antioxidants and antioxidant activity of *P.indica* dried leaves extract (n = 3)

Antioxidants/Antioxidant activity	Content
Total phenolics (mg GAE[1]/g dry weight)	84.69 ± 1.56
Total tannins (mg TAE[2]/g dry weight)	69.63 ± 0.39
Total flavonoids (mg QUE[3]/g dry weight)	45.15 ± 1.08
IC_{50}[4] (mg/ml extract)	0.04 ± 0.00
TEAC[5] (mg TE/g dry weight)	81.04 ± 0.55

[1] GAE = Gallic acid equivalent
[2] TAE = Tannic acid equivalent
[3] QUE = Quercetin equivalent
[4] IC_{50} = Concentration of antioxidants that inhibited 50% of DPPH concentration
[5] TEAC = Trolox equivalent antioxidant capacity

3.2 Cellular antioxidative stress assay

3.2.1 IC_{50} of hydrogen peroxide

Percentages of *S. cerevisiae* survival after treated with H_2O_2 at various concentrations and IC_{50} are shown in Figure 1. The IC_{50} was the concentration of H_2O_2 that killed 50% of yeast cells from oxidative stress-induced by H_2O_2. The results showed that IC_{50} of H_2O_2 was 1.88 mM. Yeast cell survival in the experimental plates are also shown in Figure 2.

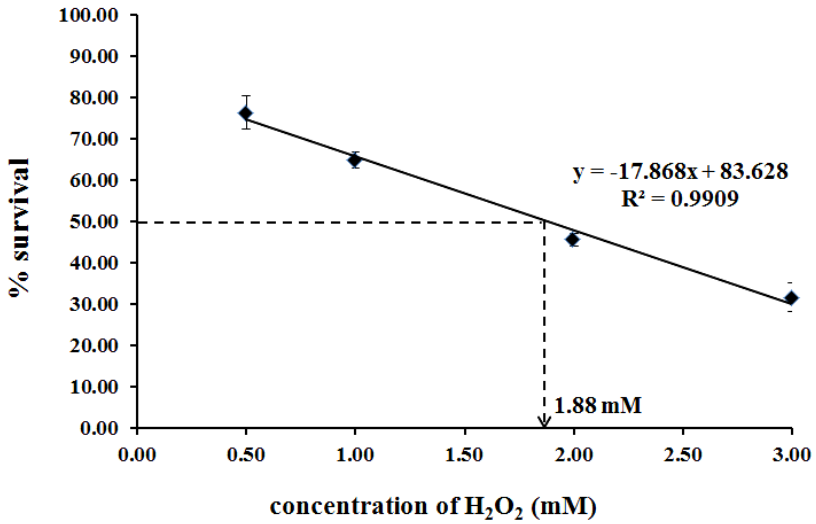

Figure 1 Percentages of survival of *S. cerevisiae* after treated with 0.50-3.00 mM H_2O_2

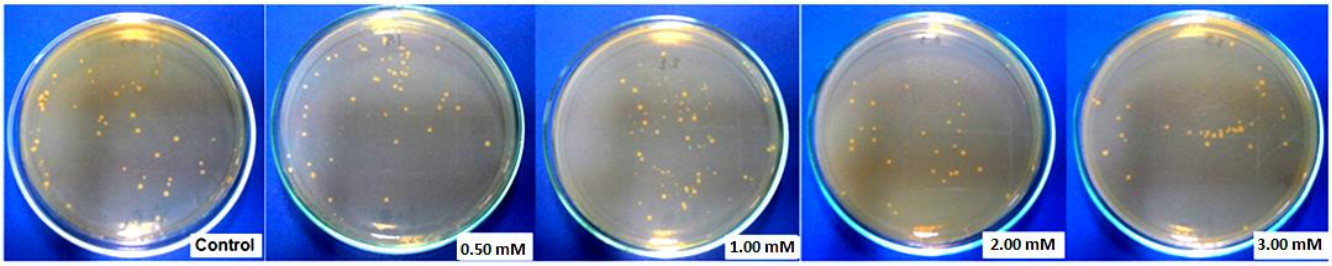

Figure 2 Survival of *S. cerevisiae* after oxidative stress induced by 0.50-3.00 mM H_2O_2

3.2.2 Antioxidative stress assay

Cellular antioxidative stress assay was performed using *S. cerevisiae* pretreated with 5.00–1,000.00 μg/ml of *P. indica* dried leaves extract, 50.00 μg/ml of standard quercetin and sterile double-distilled water as control, then H_2O_2 was added at IC_{50} concentration (1.88 mM). The results are shown in Figure 3 and yeast cell survival on the experimental plates are shown in Figure 4.

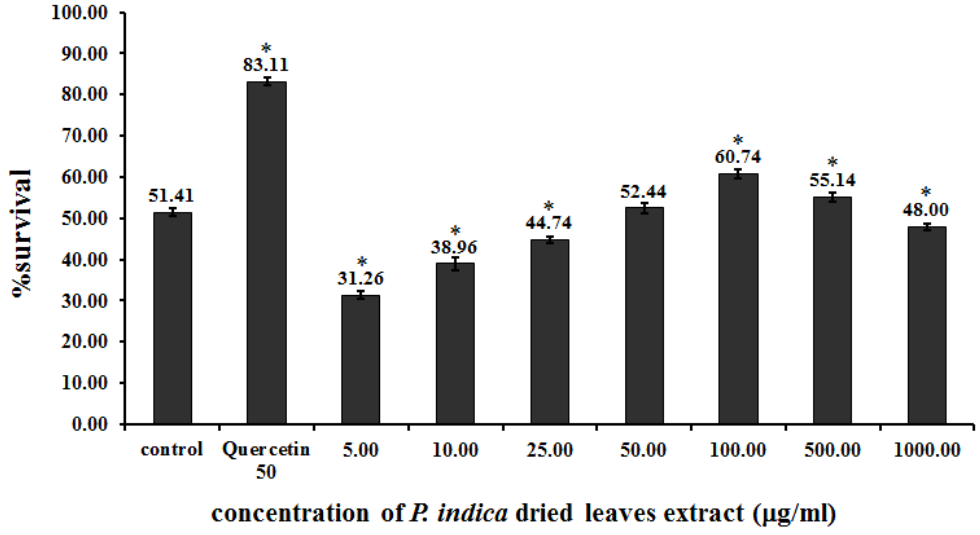

Figure 3 Percentages of survival of *S. cerevisiae* pretreated with 5.00–1,000.00 μg/ml of *P. indica* dried leaves extract, 50.00 μg/ml of standard quercetin (quercetin 50) and sterile double-distilled water (control) 1 h before exposured to H_2O_2 at IC_{50} (1.88 mM). (* represent significant difference (p<0.05) as compared to control group.)

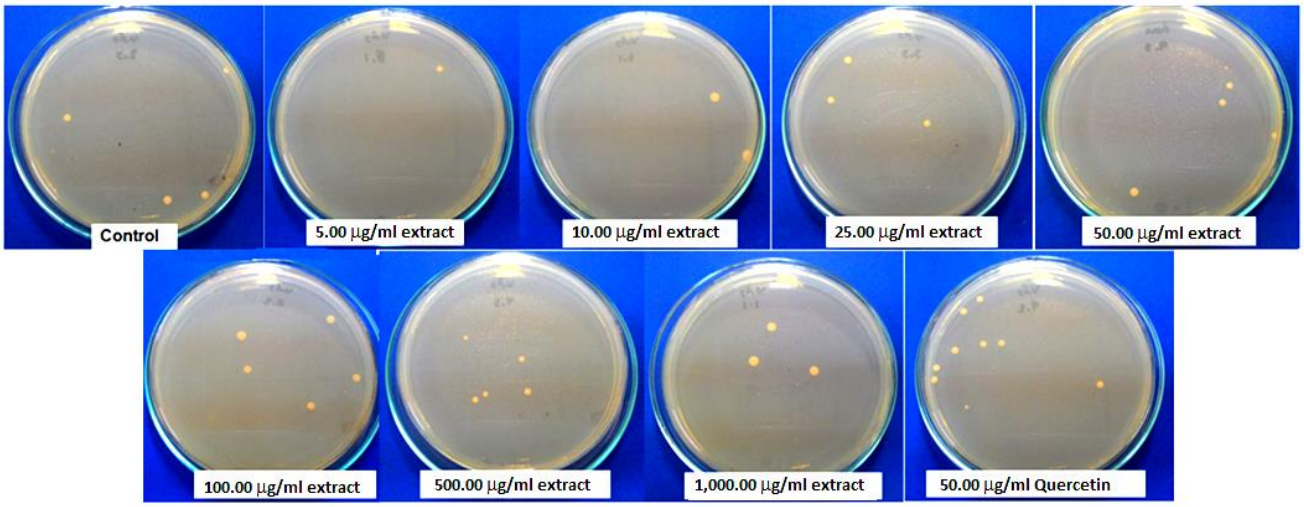

Figure 4 Survival of *S. cerevisiae* after pretreated with the extracts, standard quercetin and control, then exposed to H_2O_2 at IC_{50}

The results showed that percentages of survival of *S. cerevisiae* at stationary phase significantly increased with increasing the concentration of the extract pretreated to the cells ($p<0.05$), except at 1,000.00 µg/ml. The highest survival rate was at 100.00 µg/ml of the extract (%survival = 60.74 ±1.12) or 3/4 fold compared with that of 50 µg/ml standard quercetin (%survival = 83.11±0.89). The concentrations of the extract between 5.00–25.00 µg/ml had lower protective effect than its control significantly ($p<0.05$). It might be too low concentration to scavenge free radicals. In contrast, the concentrations of 100.00 and 500.00 µg/ml had more significant defensive activity against H_2O_2 compared with its control ($p<0.05$). At the concentration of 50.00 µg/ml of the extract, the defensive activity was not significantly different compared to its control ($p>0.05$). However, survival rate of *S. cerevisiae* was significantly lower than its control ($p<0.05$) when it was pretreated with 1,000.00 µg/ml of the extract. The results were consistent with the previous studies. Wiyakrutta et al. [13] found that Phikud Navakot extract increased the survival of *S. cerevisiae*, which was induced to be stress by H_2O_2, with increasing the concentrations (5-30 µg/ml). But the extract became toxic to the cells at the concentrations more than 50 µg/ml. The same phenomenon occurred in the effect of aqueous extract of *Rhodiola rosea* against H_2O_2 in *S. cerevisiae*. The extract showed a high viability of yeast cells at low concentration (1 µl/ml), while the extract at a higher concentration (5 and 20 µl/ml) decreased the survival rate compared to the control [20]. These results demonstrated that decreasing of cell survival at high concentration of the extract might be due to the reversed protective effect or toxicity of the extract to the cells.

4. Conclusions

P. indica dried leaves extract had high antioxidant capacity due to high concentration of major groups of antioxidants; phenolic compounds, tannins and flavonoids. These compounds were responsible for the antioxidant activity of this plant. Moreover, *P. indica* dried leaves extract also showed antioxidative stress at a cellular level or cytoprotective effects induced by H_2O_2. The increase in the protection of yeast *S. cerevisiae* from reactive oxygen species might be due to the process of free radical scavenging. Thus, herbal tea from *P. indica* may be an alternative healthy drink recommended to the consumers for preventing various diseases caused by oxidative stress.

5. Acknowledgements

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