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Phenolic compound and antioxidant levels of *Prismatomeris glabra*

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Abstract
A decoction of the roots of *Prismatomeris glabra* (PG), family Rubiaceae, has been traditionally used by rural people for wellness effects. However, there are no scientific data to support the folkloric use of this plant. This research was thus conducted to determine whether aqueous extract of PG roots possess antioxidant capacity. PG extract was prepared by boiling powdered roots for 10 minutes before drying in a spray dryer. Antioxidant capacity was determined using photochemiluminescent method, DPPH radical scavenging assay, ferric reducing antioxidant power (FRAP) assay, *tert*-butyl hydroperoxide (*tert*-BOOH) induced lipid peroxidation assay and by measurement of the end product of lipid peroxidation, malondialdehyde, produced *in vivo*. Antioxidant studies showed that PG did not possess high antioxidant capacity as compared to phenolic antioxidant standards. *In vitro*, lipid- and water-soluble antioxidant capacities of PG were 36.61±1.39 µg/mg of ascorbic acid equivalent and 8.28±1.23 µg/mg of trolox equivalent, respectively. Total phenolic content of the extract was 6.82±0.71%. PG scavenged DPPH radicals, reduced ferric ions and inhibited *tert*-BOOH-induced lipid peroxidation with values of 239.31±70.48 µg/ml (EC_{50}), 0.298 ± 0.026 µmol Fe^{2+}/mg and 188.7±15.3 (IC_{50}), respectively. *In vivo*, PG (500 mg/kg/d, p. o., acute and 7-day daily dosing) did not affect malondialdehyde levels of major organs and plasma. In conclusions, aqueous extract of PG roots possess about a third the antioxidant capacity of ascorbic acid and a tenth the antioxidant capacity of trolox, a vitamin E analogue. Thus, PG roots may not be an important source of antioxidants, although it apparently has sufficient antioxidant capacity to enhance wellness.

Keywords: *Prismatomeris glabra* (PG), antioxidant, phenol, root.

1. Introduction
Prismatomeris glabra (*P. glabra*) is a tropical plant which normally grows on hillsides and ridges of tropical forests at altitudes up to 700 m. The plant owns several traditional names, but is famously known as Aji Samat. It is occasionally used in wellness remedies. According to users of the plant, a decoction of its roots is traditionally used for various purposes such as alertness, to induce feeling of freshness and as illness medication. However, there is no scientific evidence to support the plant's folkloric use, which is a setback for future commercialization of this plant.

Antioxidant assay is one of the experimental studies that can be associated with investigation of wellness effects of a substance [1]. This is because antioxidants provide defense mechanisms against free radicals and reactive species that are formed by the body or which enter the body [2]. Free radicals are atoms or molecules which possess an unpaired electron in their outermost orbitals, making them highly reactive. Excessive generation of free radicals and reactive oxygen species (ROS) in excess of antioxidant capacity has been associated with many diseases such as atherosclerosis leading to incidence of stroke [3, 4, 5], development of cancer [6, 7], brain ischemia with consequent brain damage [8], impairment of internal organs, such as heart [9], kidneys [10], liver [11], pancreas [12] and lungs [13, 14], which may lead to the onset of cardiac dysfunction [15], kidney failure [10], liver failure [16] and diabetes [17]. Low antioxidant levels are also associated with lower resistance of the immune system and high susceptibility to infections [18]. Ability of antioxidants to protect tissues and internal organs makes them a potent class of substances in the body [19]. Hence, the search for new sources of antioxidants is being continuously made.

2. Materials and methods
2.1. Preparation of PG Aqueous Extract
PG plants were collected from the tropical jungle in the Peninsular under supervision of jungle officers.

The plants were verified by phytologists from Forest Research Institute of Malaysia (FRIM). The plant was given a voucher code of PT/UiTM/AS1 and kept in the Faculty's herbarium. Fresh roots of the plants were chipped into small pieces within 24-48 h of collection and dried at 45 ºC in the oven for three days. Dry root chips were grounded to crude powder before every 100 g of them were boiled in 1 L of distilled water for 10 minutes. The suspension from the boiling process was filtered using filter paper. The filtrate was collected and dried using laboratory spray dryer (Büchi Mini Spray Dryer B-290). PG aqueous extract powder was kept in -20 ºC freezer until use.

2.2. Antioxidant analysis

Measurement of antioxidant capacity of PG extract was done *in vitro* and *in vivo*. Measurement of antioxidant activity *in vitro* was conducted for measuring lipid-soluble and water-soluble antioxidants capacity, total phenolic content, 2,2'-diphenyl-1-picrylhydrazyl (DPPH) scavenging ability, ferric reducing antioxidant power (FRAP) and tert-butyl hydroperoxide-induced lipid peroxidation inhibition ability. Measurement of antioxidant activity *in vivo* was done by determination of MDA, a biomarker to measure the level of lipid peroxidation, in selected organs and serum of mice that were treated with PG aqueous extract.

(a) Photo chemiluminescence analysis: Lipid-soluble antioxidants (ACL) and water-soluble antioxidants (ACW) capacities were measured using a photochemiluminescence technique (Photochem®, Analytic Jena AG, Germany). Briefly, radicals are synthesized by ultraviolet radiation of a photosensitizer. These radicals are partially eradicated by antioxidants (samples). The remaining radicals are quantified by luminescence detector. The antioxidant samples were quantified by comparing luminescence generation of their inhibitory effect with luminescence generation of inhibitory effect of a standard antioxidant, e.g. ascorbic acid for ACW and Trolox for ACL [20, 21, 22].

(b) Total phenolic content analysis: Total phenolic compound was determined using Folin-Ciocalteu reagent based on an established method [23] using catechin as the standard antioxidant. This method is based on calorimetric technique by Lowry (1951) [24]. Principally, the use of Folin-Ciocalteu reagent is to react with reducing substances to form chromogens that can be detected by spectrophotometer. Phenolic content in the sample was determined from a standard curve of catechin.

(c) DPPH radical scavenging analysis: DPPH radical scavenging assay was conducted using an established protocol [25]. DPPH is a stable free radical molecule that acquires delocalized electron or unpaired electron that gives rise to the dark violet color that can be detected by spectrophotometer at the absorbance of about 520 nm [26, 27]. When a solution of DPPH is mixed with reducing agent or antioxidant, DPPH is reduced and violet color disappears and is changed to pale yellow due to the presence of picryl group [27]. Results from DPPH method can be interpreted by the EC_{50} value, an efficient concentration of substrate that causes 50% loss of DPPH activity (colour), that was used by many workers [27, 28, 29, 30]. Quercetin, trolox, gallic acid and ascorbic acid were used as standards, whereas ethanol was used as blank. Absorbance of blank was subtracted from sample or standard absorbance values to obtain the percentage inhibition.

(d) FRAP assay: FRAP analysis was performed using an established method [31]. It determines total antioxidant capacity of a sample by using antioxidants as reductants in a redox-linked colorimetric method. In principle, TPTZ (2, 4, 6-tripyridyl-s-triazine) reacts with ferrous (Fe^{2+}) to form a deep blue-purple color of ferrous tripyridyl triazine (Fe II TPTZ). When FRAP working solution is prepared by mixing TPTZ with ferric chloride, ferric tripyridyl triazine (Fe III TPTZ) is formed. At acidic pH, when antioxidant sample is added into the solution, Fe III TPTZ is reduced to Fe II TPTZ. The change of color can be detected at an absorbance of 593 nm by spectrophotometer. The ability of antioxidant in the sample/standard to reduce Fe^{3+} ($FeCl_3$) to Fe^{2+} was determined by comparing absorbance of the sample/standard to the standard curve for $FeSO_4$.

(e) Tertiary-butyl hydroperoxide-induced lipid peroxidation inhibition assay: Inhibition of tertiary-butyl hydroperoxide-induced lipid peroxidation was determined by assaying for the product of lipid peroxidation, malondialdehyde (MDA) by the thiobarbituric acid reactive substance (TBARS) method [24, 32, 33, 34]. Principally, MDA reacts with thiobarbituric acid (TBA) to form a 1:2 MDA-TBA adduct substance under acidic condition and high temperature (95 – 100 ºC). The reactive substance can be read using spectrofluorometer at excitation wavelength of 515 nm and emission wavelength of 553nm. Percentage of inhibition was plotted against sample/control concentration in graph and concentration at 50% inhibition (IC50) of sample/control was able to be determined. A serial dilution of MDA solution was used as standard. Trolox (6-hydroxy-2, 5, 7, 8-tetramethyl-chroman-2-carboxylic acid, 97%) was used as positive control. Methanol was used as blank.

(f) Determination of organ lipid peroxidation by malondialdehyde-thiobarbituric acid reactive substances (MDA-TBARS) assay: The use of laboratory animals in this study was approved by the university ethic board and all animals were treated in standard protocols. Eighteen healthy *Mus musculus* mice were selected and randomly divided into 2 groups which were labeled for control (n=9) and PG-treated (n=9) groups. Control group was treated with 0.1 ml/10 g p.o./day of 0.9% normal saline for 7 days. PG-treated group was given a dose of 500 mg/kg of PG aqueous extract by administrating the group with 0.1 ml/10g p.o./day of 50 mg/ml PG dissolved in 0.9% normal saline for 7 days. Chow and water were given ad libitum. Mice were maintained under standard condition with 12h-12h of light and dark cycle. On the 8th day, mice were sacrificed through cervical dislocation following minimal anaesthetic. While heart was still pumping, blood was immediately collected through cardiac puncture and kept into heparin tube and glass test tube. Lung, heart, liver and kidneys were harvested and weighed. Blood was centrifuged at 300 x *g* for 15 min for separation of plasma. The organs were homogenized for a few seconds before they were centrifuged at 4 ºC and for 15 min to separate the supernatant from the pellet. Supernatant was taken up and kept in -80 ºC freezer before further analysis. Determination of lipid peroxidation in these organs using MDA as marker was based on previously established method [33]. MDA plasma

was measured using high performance liquid chromatography (HPLC) based on established method [35, 36].

2.3. Statistical analysis
Data were expressed as mean ± SD. Independent-samples t-test was used for selected comparisons between samples. Alpha value was set *a priori* at $p<0.05$.

3. Results
3.1. Lipid- and water-soluble antioxidant and total phenolic content of PG
The lipid- and water-soluble antioxidant capacity of PG aqueous extract was 36.61±1.39 µg trolox equivalent/mg (n=4) and 8.28±1.23 µg ascorbic acid equivalent/mg (n=4), respectively. Total phenolic content of PG extract was 68.2±7.1 mg catechin equivalent/g (n=3).

3.2. Scavenging DPPH radical by PG
EC_{50} of PG root extract at scavenging DPPH radicals was 239.31±70.48 µg/mL. The EC_{50} of PG was compared with those of antioxidant pure compounds, namely trolox, ascorbic acid, gallic acid and quercetin. The EC_{50} values for trolox, ascorbic acid, gallic acid and quercetin were 7.65±0.50, 7.51±0.61, 7.17±1.34l, 8.43±0.69 µg/ml, respectively. EC_{50} for scavenging of DPPH radicals by PG extract was about 30 times larger than those of the pure antioxidant compounds, indicating that PG was much less potent than these pure compounds.

3.3. Ferric reducing antioxidant power (FRAP) of PG
Ferric reducing antioxidant power (FRAP) of PG root extract was compared to those of pure compounds, viz, trolox, gallic acid and curcumin at same concentration of 100 µg/ml. FRAP values for PG (0.298±0.026 µmol Fe^{2+}/mg) was very low when compared to reducing power of gallic acid (16.565±0.695 µmol Fe^{2+}/mg), trolox (7.461±0.421 µmol Fe^{2+}/mg) or curcumin (4.723±0.325 µmol Fe^{2+}/mg).

3.4. Inhibition of tertiary-butyl hydroperoxide induced lipid peroxidation by PG
PG extract elicited concentration-dependent inhibition of tertiary-butyl hydroperoxide (*tert*-BOOH)-induced lipid peroxidation. A concentration of 1000 µg/mL elicited more than 85% inhibition of *tert*-BOOH-induced lipid peroxidation. Percentage inhibition of *tert*-BOOH-induced lipid peroxidation of between 20 to 40% was achieved with PG concentrations of 10 and 100 µg/mL, respectively. Median inhibitory concentration (IC_{50}) of PG was compared to those of antioxidant standards, trolox and quercetin. PG extract was not as potent as trolox or quercetin at inhibition of lipid peroxidation induced by *tert*-BOOH. IC_{50} values of trolox and quercetin were about 20 and 90 fold smaller than that of PG root extract.

3.5. *In vivo* antioxidant assay of PG
To determine if PG extract exerted antioxidant activity *in vivo*, mice were sub-acutely dosed with the extract (500 mg/kg, p.o./day) for 7 days. Levels of malondialdehyde (MDA), a short chain aldehyde which is a product of lipid peroxidation, were measured in organs after 7 days of repeated treatments. The data showed MDA of control and PG-treated groups to be similar. In both groups, highest MDA level was seen in heart followed by lung, kidney and liver (Figure 1). Even though MDA in kidney, heart and lung of controls appeared higher than those of PG-treated group, the difference was not significant. Plasma MDA levels of both control and PG-treated groups after daily treatment for 7 days were also similar (Figure 2).

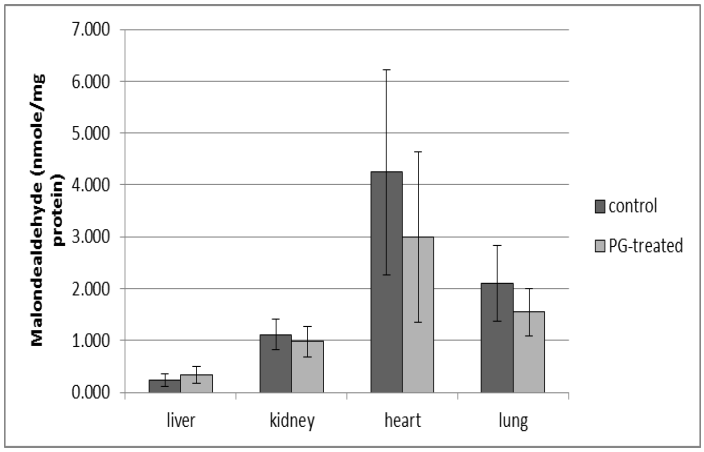

Fig 1: MDA level (nmol/mg protein) in liver, kidney, heart and lung of control and PG-treated mice after daily treatment for 7 days. Mean±S.D. (n=9 per group). No significant difference between groups (p>0.05, unpaired t-test).

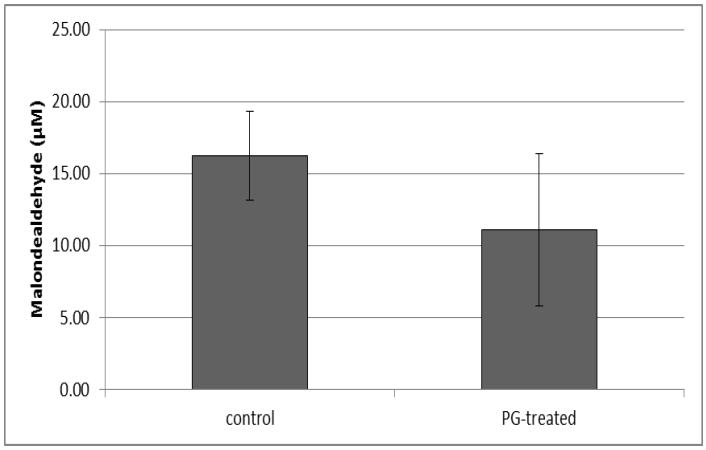

Fig 2: Plasma MDA level of the control and PG-treated mice after daily treatment for 7 days (sub-acute). Mean±S.D. (n=9 per group). No significant difference between groups (p>0.05, unpaired t-test).

4. Discussion
Lipid-soluble antioxidant capacity of PG aqueous extract was 36.61±1.39 µg trolox equivalent (TE)/mg, whereas its water-soluble antioxidant capacity was 8.28±1.23 µg acid ascorbic equivalent (AAE)/mg. Both values were expressed based on dry weight of PG extract. However, when both values were recalculated based on dry weight of PG roots, they were 20 fold lower than the respective values based on dry weight of extract. From the process of preparing a decoction of dry PG roots, approximately only 70% of aqueous solution was recovered after filtration. This filtrate was spray dried which resulted in recovery of approximately 3.5% of dry powder. If these antioxidant capacities are expressed in mmol/100 g, lipid soluble and water soluble antioxidant capacities would be equivalent to 0.731 mmol/100 g and 0.235 mmol/100 g of dry root, respectively. When these values are combined, total antioxidant capacity of PG root is 0.966 mmol/100 g of dry

root. A comparison of this value to antioxidant capacities of other plant sources such as cereals, vegetables and fruits [37, 38] showed that the antioxidant capacity of PG root to be fair.

Dried roots of PG were boiled at a concentration of 100 g per litre of distilled water for extraction.

Thus, lipid- and water-soluble antioxidant capacities of PG can also be expressed as 0.731 and 0.235 mmol/L, respectively. Antioxidant content equivalent to ascorbic acid (AAE) of PG root aqueous extract was four times lower than antioxidant capacities of various green teas, but similar to Chinese oolong and African roibos [39].

Total phenolic content of PG root aqueous extract equivalent to milligram of catechin (catechin equivalent, CE) was 68.2±7.1 mg/g of sample or 6.82±0.71% of CE in weight per weight percentage. Based on dry root weight, total phenolic content was 3.4±0.4 mg CE/g or 0.34±0.04%. There was no previous data on phenolic content of PG roots. PG total phenolic content that was expressed per 100g of dry root, was 340 mg CE. This figure is much higher than that of water extract of tropical fruits such as pineapple, banana (*Pisang mas*) and guava and other sources [41, 42, 43]. Phenolic content can be categorized into high, moderate or low at values of 486, 133 or 10 mg/kg, respectively [40]. Based on this category, phenolic content of PG is considered high.

DPPH is a stable radical and in the presence of a substance that can donate an electron or a hydrogen atom, i.e. an antioxidant, DPPH is changed to non-radical form [26]. This is shown by a change in colour from purple to yellow and can be measured by a decreased in absorbance at 517 nm. In alcoholic solution, DPPH radical (purple coloured) absorbance which can be read at 517 nm, disappears when antioxidants scavenge the radicals by donating hydrogen atoms to become a stable diamagnetic molecule of diphenyl-picrylhydrazine (yellow colour), thus allowing determination of antiradical power of antioxidants [44, 45]. The median effective concentration value, EC_{50}, is used to indicate efficacy of antioxidant scavengers of DPPH radicals. EC_{50} is defined as the concentration of substrate that causes 50% loss of the DPPH activity [27] and is used by many [46, 47, 48].

PG inner root skin is orange in colour. When PG roots were boiled, the colour of the decoction ranged from dark orange to brown. Reconstitution of samples of aqueous extract of PG produced solutions that were normally orange in colour. Therefore, during assay, colour factor of sample was subtracted by use of a colour blank at each PG concentration. EC_{50} of PG root extract for inhibition of DPPH radicals was 239.31±70.48 µg/mL. Effect of PG on scavenging DPPH was compared with that of antioxidant pure compounds i.e. trolox, ascorbic acid, gallic acid and quercetin (Table 4). EC_{50} of PG root extract was about 30 times larger than those of antioxidant pure compounds, indicating that its potency as scavenger of DPPH radicals was about 3 folds lower. However, when DPPH radical scavenging activity of PG root extract was compared to other antioxidant sources [48, 49, 50, 51, 52, 53, 54, 55], PG shows comparable ability of scavenging activity.

Even though DPPH is a stable radical, its stock solution can slowly deteriorate [26]. To overcome this, the assay is best conducted under a nitrogen atmosphere and if a burette is used, it should be covered with aluminium foil [27]. This should reduce loss of DPPH free radicals up to about 2 – 4 percent a week [27]. To overcome difficulties associated with decomposition of DPPH stock solution, freshly prepared solutions were used. Furthermore, DPPH assay was done under dim-light and test tubes were covered with aluminium foil. DPPH was incubated with samples for 10 minutes. This incubation period was within the range of reaction times (from 5 to 30 min) that were practiced by previous workers; 5 min [28]; 10 min [29]; 30 min [26, 30]. Reaction time is influenced by types of substrates and antioxidants [56, 57].

FRAP assay is the only assay that directly measures reducing ability of a sample in a redox-linked colorimetric reaction. An antioxidant is an electron-donating reductant, thus FRAP assay was used to determine reducing power of an antioxidant substrate [37]. In this assay, colourless ferric ion (Fe^{3+}) was oxidized by antioxidant to form ferrous ion (Fe^{2+}) which later formed blue-coloured Fe^{2+}/TPTZ complex. Colour absorbance of the complex was read at 593 nm, it may change according to reduction intensity of sample [42]. Ferric reducing power of PG root extract was 0.298±0.026 µmole Fe^{2+}/mg or 29.8±2.6 mmole Fe^{2+}/100g of dry weight. FRAP value of PG root extract was about a fold lower than of curcumin and about 20 and 50 times lower than trolox (vitamin E analogue) and gallic acid, respectively. When FRAP value of PG was recalculated based on raw sample, FRAP was equivalent to 1.49 mmole Fe^{2+}/100 g dry weight of roots, which is comparable to other sources of antioxidants [37, 38, 42].

Aqueous extract of PG roots demonstrated more than 85% inhibition of lipid peroxidation at 1000 µg/mL. From concentration-response curves, trolox elicited 50% inhibition (IC_{50}) of lipid peroxidation at 8.4±0.7 µg/mL while IC_{50} for PG was 188.7±15.3 µg/mL. Inhibition of lipid peroxidation by PG was concentration-dependent. Although not as potent as trolox at inhibiting lipid peroxidation, PG demonstrated comparable effects to other natural sources [2, 49, 53].

PG root extract was administered sub-acutely (daily treatment for 7 days) to mice for determination of antioxidant activity *in vivo*. Mice were dosed every day with PG at 500 mg/kg body weight p.o.. Following subacute dosing, mice were killed and malondialdehyde (MDA), a short chain aldehyde which is an end product of lipid peroxidation was measured in organs using the thiobarbituric acid reducing substances (TBARS) assay. This assay is a valid measure of MDA as most of TBARS is MDA and can be efficiently determined using spectrophotometry or spectrofluorometry [58, 59]. TBARS levels in plasma and organs of PG-treated mice were not significantly different from control following sub-acute treatment. TBARS levels in organs and plasma of the control group was only slightly higher than those of PG-treated group giving no indication that PG had a protective effect in organs. However, since animals used in this study were not induced with oxidative stress by use of pro-oxidative agents, the actual protective effect afforded by PG extract was not truly studied.

Amongst villagers, the traditional way of taking PG is to first cut freshly collected roots into small chips which are then dried for a few days in the sun. Infusion of dried root chips is prepared by boiling for several minutes. The liquid obtained is filtered before it is taken. Normally the root chips are reboiled and reused until the brownish colour of the filtrate has disappeared. To prepare an aqueous extract of PG roots, this traditional process was followed except that the filtrate was immediately dried prior to storage. Furthermore, root chips were not reused.

Usage of PG roots as a traditional remedy for maintenance of health and for wellness is most likely associated with its antioxidant capacity which was first time measured in this study. PG roots have higher antioxidant capacity when compared on a weight to weight basis to many fruits and vegetables. Total antioxidant capacity of dried, freshly

collected roots was comparable to those of many vegetables and was higher than some fruits. Preparation of aqueous extract of PG roots resulted in enhancement of total antioxidant capacity as the extract was about 21 times more active than the dried, freshly collected roots. Green tea is a well known source of antioxidants [60]. In comparison to green tea, antioxidant capacity in terms of ascorbic acid equivalents of the dried, freshly collected roots was about 2 magnitudes lower (~ 0.2 vs 20 mmol AAE/L). Total phenolic content is a measure of reducing power [61, 62, 63]. Total phenolic content of PG was about a magnitude higher (340 vs ~ 30 mg CE/100 g) than those of common beverages viz tea, coffee or cocoa. In terms of DPPH radical scavenging, aqueous extract of PG roots showed comparable activity to methanolic extract of herbs (*C. asiatica*, *T. foenumgraceum*, *P. alba*), about a magnitude lower scavenging activity of *P. niruri* (200 vs ~ 20 µg/mL) and about half that of roots of *P. grandiflora*. Another measure of reducing power as an indicator of antioxidant activity is determined via FRAP assay. FRAP value of aqueous extract of PG roots was about 2 magnitudes lower than those of reference pure compounds (0.3 vs 7 or 5 µmole Fe^{2+}/mg). On the other hand, FRAP value of aqueous extract of PG roots was 2 magnitudes higher than those of tea/coffee/cocoa (30 vs 0.3 µmole Fe^{2+}/mg) and a magnitude higher than those of vegetables (30 vs 3 or 2 µmole Fe^{2+}/mg).

A reference standard of antioxidant activity is lipid peroxidation inhibitory activity [64, 65]. Lipid peroxidation is a condition whereby lipid is oxidized by radicals or reactive oxygen species leading to oxidative stress and cell damage [66]. In absence or reduced capacity of protective mechanisms i.e. antioxidants, pro-oxidant effects predominate leading to development of oxidative stress [67]. In the laboratory, lipid peroxidation can be induced by many chemicals and can be seen in many types of tissue injuries [68]. In this study, tert-butyl hydroperoxide was used as lipid peroxidation inducer in rat liver microsomes. PG root extract demonstrated high protective ability against peroxidation at 1000 µg/mL. Lipid peroxidation inhibitory activity of aqueous extract of PG roots was comparable to those of pure flavonoid compounds of *R. alaternus* and twice the activity of extracts of *P. alba, S. androgynus* and *D. hamiltonii* roots. In comparison to reference antioxidant pure compounds i.e. trolox and vitamin C, PG was about 23 to 12 times, respectively, less active at inhibition of microsomal lipid peroxidation.

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