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Generation of free radical intermediates from traditional medicine herbal extracts

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ABSTRACT

Varieties of medicinal plants are well-known to possess powerful antioxidants and often considered for use as cancer preventing agents. Dietary supplements from plant extracts are made available for the purpose of scavenging free radicals. However, due to their strong reactive nature, herbal extracts may also produce free radicals which lead to other effects such as cell killing as observed in studies showing tumor cell death caused by herbal treatments. In this study, we examined a range of herbs commonly used in traditional medicine for their ability to generate radical intermediates in both aqueous and ethanolic extracts using Electron Paramagnetic Resonance (EPR) spectroscopy. Crude aqueous and ethanolic extracts of *Phyllanthus emblica* Linn, *Phylanthus urinaria* Linn, *Houttuynia cordata* Thumb, *Acanthus ebracteatus* Vahl, *Rhinacanthus nasutus* Kurz, *Thunbergia laurifolia* Linn, and *Moringa oleifera* Lam were tested for their ability to generate free radicals under different conditions. The results showed that all of the herbal extracts could act as prooxidants under extreme alkali condition (pH12); *P. emblica*, *P. urinaria* and *A. ebracteatus* extracts were able to produce free radical in phosphate buffer at near neutral pH (pH7.4). At near physiological conditions where H_2O_2 and peroxidase are available, *H. cordata* in addition to the three herbs mentioned prior was able to generate a free radical product. The HPTLC data also showed that gallic acid, chlorogenic acid and quercetin were present as constituents of these plants and thus could play a role in their free radical production. This study provides evidence that herbal extracts can produce free radicals and cause cellular toxicity rather than simply scavenging free radicals.

INTRODUCTION

A number of traditional medicines are now being used as alternative treatment modalities for patients. Many of these medicines have been used to treat symptoms and diseases for centuries. These medicinal plants contain phytochemical compounds which, mostly, have antioxidant properties. However, during the metabolism of these polyphenolic compounds, reactive free radicals can be generated (Galati *et al.,* 2002). This physiological context is often overlooked as polyphenolics are generally regarded as antioxidant agents. Gallic acid, which has been used as a reference for phenolic content as well as antioxidant activity, appears to be able to generate free radical at pH 7 and above (Eslami *et al.,* 2010). This observation leads to many questions of physiological functions of phenolic constituents in plants in human health. Although a number of plants have been demonstrated to provide beneficial effects against oxidative stress-related diseases, some of them appeared to have null effect (Martins *et al.,* 2016). The production of free radical by phenolic compounds has also been previous reported in vitro for cell culture experiment (Long *et al.,* 2000). Free radical generation could undoubtedly alter cellular redox state and signaling as well as homeostasis of cells treated with these plant extracts. Despite the vast number of plants that have been tested for their phenolic contents and their antioxidant activity (Cai *et al.,* 2004; Kahkonen *et al.,* 1999; Kim *et al.,* 1997),

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very limited studies have shown their potential in acting as prooxidants and causing free radical formation. Therefore, in this study, we addressed whether some of the major medicinal plants used in traditional medicine could act as prooxidants at functional pH's which could provide the basis for their effective use as traditional medicines.

MATERIALS AND METHODS

Plant specimen collection and preparation of botanical extracts

Plant specimens were purchased from local pharmaceutical stores. An aqueous crude extract was achieved as follows. The leaf parts were air dried at room temperature and crushed into a fine powder using a blender. The extract was prepared 1:10 (w/v) in boiling distilled water for 10 min and left at room temperature for 1 hour before being paper filtered (Whatman Filter Paper No.4). The extract was freeze-dried (ScanVac, Denmark) and kept at -20°C until use. For ethanolic extraction, dry powders of plant materials were extracted in 95 % ethanol at room temperature for 24 hour then filtered. The ethanol in the filtrates was removed by rotary evaporator and the extracts were kept at -20 °C. Phosphate-buffered saline and DMSO were used to dissolve the aqueous and ethanolic extracts, respectively.

Quantification of phenolic contents

The total soluble phenolic content of the crude extract was quantified using Folin-Ciocalteu's phenol reagent and external calibration with gallic acid. Briefly, 10 mg of crude extract was prepared in 10 ml water. Then a 20 µl aliquot of the mixture was thoroughly mixed with 1.58 ml of water and 100 µl of Folin-Ciocalteu reagent and allowed to incubate at room temperature for 5 minutes. A sodium carbonate solution (20%) was added, mixed, and incubated for 90 minutes at room temperature. The absorbance was measured at 756 nm. A standard calibration curve was prepared by measuring the absorbance of a series of standard gallic acid concentrations in water: 5, 10, 25, 50, and 100 mg/ml. The unit was expressed as g of gallic acid equivalents/g dry weight extract.

Electron paramagnetic resonance spectroscopy

To determine whether the extracts were able to generate radicals, EPR spectroscopy was used. This technique involved a change in pH of the samples and/or additional reagents to form an EPR detectable radical product. The intensity of the EPR signal corresponds to the amount of short-lived radicals detected. EPR spectra were recorded using a Bruker EMX spectrometer equipped with a high-sensitivity cavity. The spectra were obtained as an average of 4 scans with a modulation amplitude of 1 G; scan rate 80 G/81 s; receiver gain $2x10^5$; microwave power 20 mW; and modulation frequency of 100 kHz. The EPR peak heights were presented in arbitrary units and were obtained at room temperature. The extract was prepared as 1 mg/ml in glass vial using water or 100 mM potassium phosphate buffer (KPB). All solutions above neutral pH (alkaline solutions) were prepared by adjusting with 5 M sodium hydroxide solution.

HPTLC analysis

HPTLC was performed using TLC silica gel 60 F254 aluminum plates (20 cm × 10 cm, Merck, Darmstadt, Germany) with chloroform: methanol: water [25:15:1 (v/v)] as a mobile phase. The standard (gallic acid, quercetin, rutin, chlorogenic acid and rosmarinic acid) solutions (2.0 µL of each concentration 1 mg/mL) and the extract solution (10 µL of each concentration 2 mg/mL) were applied as a 5 mm band onto a TLC plate using a CAMAG Linomat 5 (Camag, Muttenz, Switzerland). The plate was developed to 70 mm in Twin trough glass chamber and the developed plate was visualized under UV 254 nm. Aluminium chloride reagent (1% ethanolic solution of aluminium chloride) was sprayed to TLC plate and visualized under UV 366 nm to detect the phenolic compounds and flavonoids. The chromatogram was obtained by a CAMAG TLC Scanner 4 (S/N 190309) in absorbance mode at 254 nm with the winCATS software. The slit dimensions were 4.00 mm × 0.30 mm, and the scanning speed was 20 mm/s.

RESULTS AND DISCUSSION

In spite of the abundance of phenolic compounds within plants, under oxidative stress, free radicals, particularly hydroxyl radical, can be generated within plants through the Fenton reaction utilizing Fe ion which is a constituent of many antioxidant enzymes including catalase, ascorbate peroxidase, glutathione peroxidase and ferro-superoxide dismutase (Becana *et al.*, 1998). Thus, the addition of plant extracts into mammalian cells could affect the cells more than simply adding antioxidant reagents. In this study, we first quantified amount of phenolic contents within the medicinal plants of interest which include *Acanthus ebracteatus* Vahl (AE), *Houttuynia cordata* Thumb (HC), *Moringa oleifera* Lam (MO), *Phyllanthus emblica* Linn (PE), *Phylanthus urinaria* Linn (PU), *Rhinacanthus nasutus* Kurz (RN) and *Thunbergia laurifolia* Linn (TL). As shown in Fig. 1, PE and PU appeared to have the most phenolic constituents for aqueous and ethanolic extracts, respectively, among the plants tested.

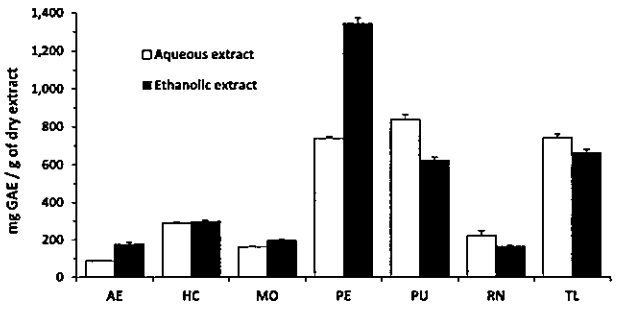
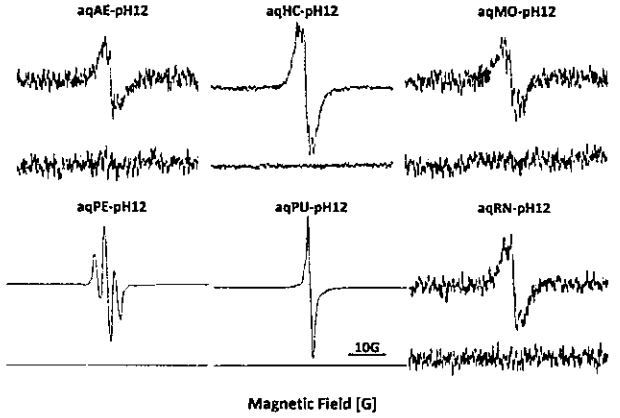
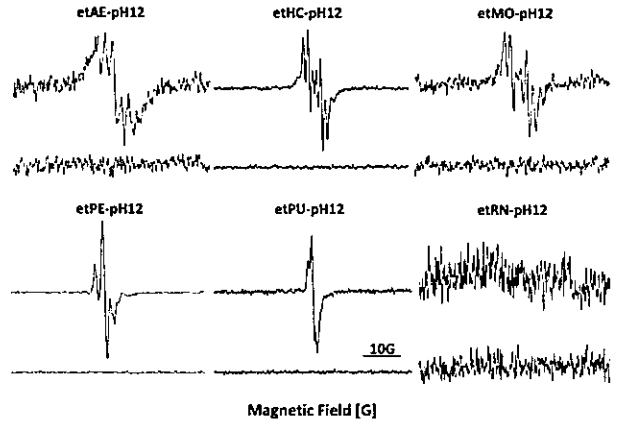


Fig. 1: Total phenolic content of crude extracts from plants expressed as mg of gallic acid equivalent (GAE) per gram of dry extract determined by Folin-Ciocalteu Method. Values represent the mean ± standard deviation.

However, the amount of phenolic compounds may not reflect the capability of free radical generation or antioxidant capacity of the plant extracts.



A



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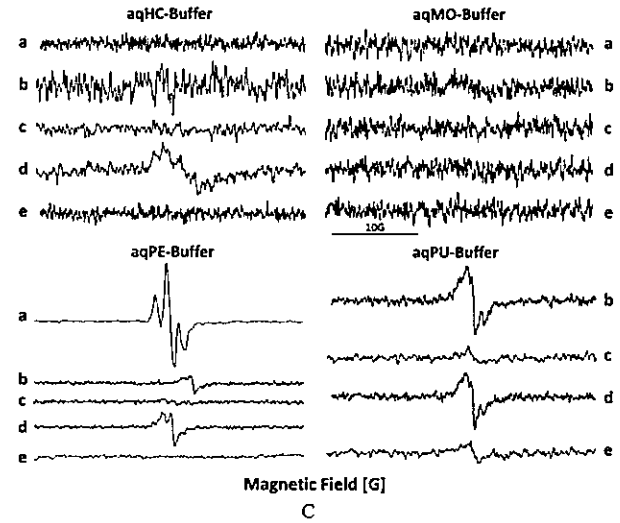

C

Fig. 2: EPR peaks of different herbal extracts in (A) alkali water (pH12) and (B) alkali ethanol (pH12) and (C) buffer conditions (a. pH10; b. pH 7.8 + HRP/H_2O_2; c. pH7.8 + HRP; d. pH7.4 + HRP/H2O2; e. pH7.4). The concentrations of HRP and H_2O_2 were 50 mM and 100 mM, respectively. The baseline plots were measurements of extracts in water or ethanol (aq – aqueous; et – ethanolic).

We proceeded to determine whether each extract could generate free radicals using EPR. Expectedly, as shown in Fig. 2A, 2B, S1 and previously shown (Jetawattana *et al.,* 2015), at the extreme alkali condition, pH12, all extracts, both aqueous and ethanolic, were able to generate free radicals due to its reductive condition. For aqueous extracts, at pH10, only PE and PU were able to produce free radical signal. However, at pH7, none of them produced a detectable free radical product. Importantly, at near neutral pH (pH7.4 and 7.8) where hydrogen peroxide and horse radish peroxidase (HRP) were present, conditions intended to mimic potential cellular environment, PE and PU continued to produce free radical products. HC and TL only provided weak signals under such conditions (Jetawattana *et al.,* 2015); whereas in the absence of H_2O_2, HRP failed to induce the same effect. Therefore, it is likely that this similar free radical inducing phenomenon would occur when herbal extracts such as PE and PU are given to cells. Generated free radicals would then trigger cellular responses and potential cellular damage if a large number of free radicals were generated which could lead to oxidation of cellular compounds. Anti-proliferative effects were also previously observed in tumor cell lines treated with plant extracts with high antioxidant activities (Lin *et al.,* 2010; Wu *et al.,* 2013). The two plants were also previously shown to have anti-tumoral effects which free radical generation could be one of the underlying mechanisms (Huang *et al.,* 2010), as tumor cells may be susceptible to pharmacological reactive oxygen species (Trachootham *et al.,* 2009).

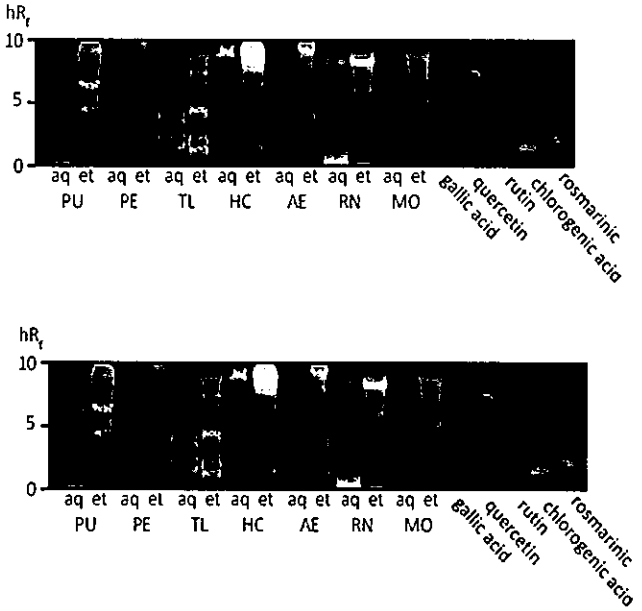

Fig. 3: HPTLC fingerprints of plant extracts and standard compounds. Visualization was under UV 254 nm (Upper panel) and 366 nm after spraying with aluminium chloride reagent (Lower panel).

We further performed HPTLC to screen for chemical constituents in the plants. Gallic acid was found to be a major component in both PU and PE extracts either in aqueous or ethanolic extracts (Fig. 3A). The presence of gallic acid was confirmed as a band with dark blue color in long wavelength UV light when sprayed with aluminium chloride reagent (Fig. 3B). Gallic acid is a prevalent compound in the plants belonging to the genus Phyllanthus and several plants in this genus and has been demonstrated to exhibit both antioxidant and prooxidant activities (Mao *et al.*, 2016; Sakagami and Satoh, 1997). Thus, gallic acid found in PU and PE might be responsible for the EPR peak. Chlorogenic acid and quercetin were also observed in TL, AE, RN and MO.

Chlorogenic acid but not quercetin was observed in HC. Chlorogenic acid is a phenolic compound with biological effect mostly related to antioxidant activity. It is mainly found in coffee and tea (Farah *et al.*, 2008; Sato *et al.*, 2011). Quercetin is a flavonoid that is considered to be a strong antioxidant found in various fruits and vegetable (Boots *et al.*, 2008). These compounds might have been the cause of the free radical intermediate generations observed in these medicinal plants as many antioxidants can display prooxidant behavior depending on their concentrations and neighboring molecules (Carocho and Ferreira, 2013). For example, a grape seed polyphenol, proanthocyanidins, can induce cardiomyocyte toxicity as a prooxidant (Shao *et al.*, 2003). A closer look and careful observations concerning free radical induction and signaling are encouraged when applying strong antioxidant or herbal extracts to cellular or disease models.

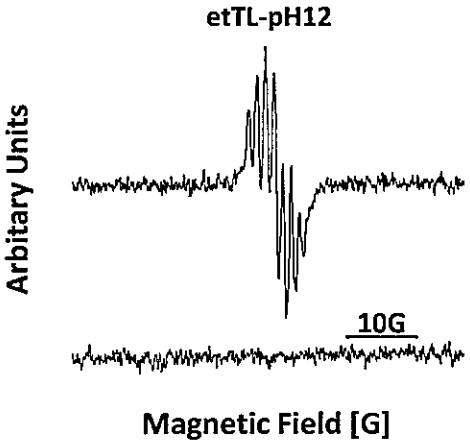

Fig. S1. EPR peak of *Thunbergia laurifolia* Lin (TL) ethanolic extract in alkali water (pH12). The baseline plots were measurements of extracts in ethanol.

CONCLUSIONS

In this study, we determined that a number of plant extracts believed to be strong antioxidants can behave as prooxidants due to their highly reductive nature under both alkali and mimicked physiological condition buffers. Gallic acid as well as chlorogenic acid and quercetin, known to be powerful antioxidant, contained within these plant extracts could be the cause of this free radical generation. Administration of herbal extracts to both in vitro and in vivo experiments, especially on studying of antioxidant, free radical and redox signaling should take prooxidant nature of plant extracts into consideration.

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Conflict of Interests: There are no conflicts of interest.

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