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Original Article

Chemical- and Cell-based Antioxidant Capacity of Methanolic Extracts of Three Commonly Edible Plants from Zingiberaceae Family

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ABSTRACT

Background: Edible plants belonging to the Zingiberaceae family display various antioxidative properties and widely used as folklore medicines. Three edible plants in Zingiberaceae family; namely *Boesenbergia rotunda*, *Phaeomeria imperialis* and *Zingiber officinale* were selected for secondary metabolites extraction using methanol. All crude extracts were investigated to evaluate their phenolics and flavonoids contents and further compare their antioxidant properties. **Methods:** Total phenolic content and total flavonoid content were evaluated using Folin-Ciocalteu method and Aluminium Complexation Reaction respectively. Conventional DPPH (2,2-diphenyl-1-picryl-hydrazyl), revolutionary CUPRAC (cupric ion reducing antioxidant capacity) and cellular antioxidant activity (CAA) *in vitro* assays were employed to evaluate the antioxidant activities of methanolic plant extracts for the first time. **Results:** DPPH and CUPRAC antioxidant assays resulted in similar trend to total flavonoid content in the order *Z. officinale* >*P. imperialis*>*B. rotunda* where as *P. imperialis* revealed highest phenols content and displayed highest CAA. **Conclusion:** Methanolic extract of *Z. officinale* showed highest free radical scavenging ability and hence flavonoids present may potentially act as natural source of antioxidant.

Key words: Antioxidant, CUPRAC, DPPH, CAA, Zingiberaceae.

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INTRODUCTION

Human metabolism (oxidative phosphorylation) generates endogenous free radicals and reactive oxygen species (ROS), giving rise to oxidative stress[1] which will then give rise to chronic diseases, such as heart disease, neurodegenerative diseases and cancer.[2] Upon ROS formation, lipid peroxidation can occur; leading to formation of toxic peroxides. These series of reactions cause direct tissue damage. ROS can also source nitrosation and deamination of amino groups in DNA point mutations and potentially, tumor formation.[3,4] Antioxidants are molecules that inhibit oxidation of other molecules. They scavenge free radicals by donating an electron, in order to pair up the unpaired electron. They are essentially reducing agents.[5] For centuries, edible plants are commonly used in traditional remedy to treat a variety of diseases.[6] Increasing antioxidants in the body will reduce ROS numbers and lower the risk of developing the aforementioned diseases. Consumption of foodstuffs containing antioxidants has proven effective in increasing plasma antioxidant concentration.[7]

There are approximately 1500 known species in Zingiberaceae family and the Malesian region contains 650 of those species.[8] They grow in tropical and subtropical areas, which are damp, humid and shady. Delin and Larsen describe these plants as, "Herbs perennial, terrestrial, rarely epiphytic, aromatic, with fleshy, tuberous or non-tuberous rhizomes, often with tuber-bearing roots. Stems are usually short, replaced by pseudostems formed by leaf sheaths.".[9] Zingiberaceae family was selected for investigation based on several reasons. Sabli *et al*. reported the ability of the genus *Etlingera* and *Zingiber* to scavenge free radicals and act as natural antioxidants.[10] A host of publications have demonstrated the antioxidant activity of *Z. officinale*. DPPH assay has shown a radical inhibition percentage of over 50% for the extracts of rhizomes from two varieties of *Z. officinale*.[11] *Z. officinale* has also been shown to reduce lipid peroxidation as much as the natural antioxidant ascorbic acid.[12] The antioxidant potential of these three edible plants have been looked into many times previously, with positive results in several antioxidant assays, such as ABTS decolourisation, FRAP assay and DPPH assay.[13] ORAC assay has also been tested on the rhizomes which reported high antioxidant activity.[14]

In 2004, Apak *et al*. successfully employed cupric ion reducing antioxidant capacity (CUPRAC) chromophore called the Cu(I)-neocuproine (Nc) chelate which formed when undergoing redox reaction with antioxidant to evaluate antiradical activity in plant extracts. This electron-transfer based assay measures the capacity of antioxidant in reduction of an oxidant which is substantiated by colour change.[15] Yildiz *et al*. solely used CUPRAC assay to associate flavonoid content with antioxidant functions.[16] A relatively new antioxidant assay, Apak *et al*. reported various advantages of CUPRAC assay over other conventional antioxidant assays. One that distinguishes it from others is the effectiveness of CUPRAC assay in quantification of total antioxidants directly from plant and food extracts. The chromogenic radical reagent is versatile and generally stable, applicable to almost all antioxidants detection in plant extracts.[17]

Antioxidant activity can also be evaluated using biological assay where by the activity is being measured *in vitro*. Cellular antioxidant activity (CAA) assay is a cell-based assay employed to measure the antioxidant activity within a cell. A fluorogenic dye is used to quantify the reactive oxygen species (ROS) within the cell cytosol.[18]

Therefore, current research aimed to quantify phenol and flavonoid content and to evaluate the antioxidant activity using conventional DPPH free radical scavenging, revolutionary CUPRAC assay alongside

with cellular antioxidant activity (CAA) assay in three commonly consumed plants in the family of Zingiberaceae.

MATERIALS AND METHODS

Plants and Chemicals

Z. officinale, B. rotunda and *P. imperialis* were obtained from Pasar Tani, Prima Saujana, Kajang, Malaysia (3°0'25"N, 101°48'26"E). The following chemicals are of analytical reagent grade and were supplied from the corresponding sources: DPPH (2,2-diphenyl-1-picryl-hydrazyl), neocuproine (2,9-dimethyl-1,10-phenanthroline), Folin-Ciocalteu reagent, ascorbic acid, BHA (Butylated hydroxyanisole), gallic acid and quercetin: Sigma Aldrich (Steinheim, Germany); sodium carbonate, monopotassium phosphate (KH_2PO_4), disodium phosphate (Na_2HPO_4) and aluminium trichloride: R&M Chemicals (Selangor, Malaysia); Methanol and ethanol: RCI Labscan (Mueng Samutsakorn, Thailand); Copper (II) chloride dihydrate ($CuCl_2.2H_2O$), ammonium acetate: Merck (Darmstadt, Germany) and hydrogen peroxide (30% v/v); Systerm® (Selangor, Malaysia); OxiSelect Cellular Antioxidant Activity (CAA) Assay Kit (Cell Biolabs, Inc.).

Apparatus

Absorption measurements were made either in quartz cuvettes using a Biochrom (Libra S12) UV-vis spectrophotometer (Cambridge, United Kingdom) or in a Varioskan Flash plate reader from Thermo Scientific (Massachusetts, USA). Rotary evaporation was performed in a Rotavaptor-210 evaporator (BUCHI, Switzerland).

Cell line

Human nasopharyngeal carcinoma (HK-1) cell line was material transferred upon signing of collaboration with Department of Anatomy, The University of Hong Kong through local collaboration with Institute of Medical Research (IMR) Malaysia.

Plant Extraction

All three plants were left to dry for one week, after which were pulverized, subjected to maceration in methanol for 3 days, in a 1:10 ratio (1 g in 10 mL of solvent). After maceration, each extract was filtered through Whatman filter paper and the solvent evaporated via rotary evaporation at 37°C[19] to yield oily plant crude extracts. Each extract was kept in -4°C for further bioassays.

Total Phenolic Content

100 µL of stock extract was placed in a test tube. 2 mL of 2% (w/v) sodium carbonate (Na_2CO_3) was added and was mixed vigorously. While mixing, 100 µL of 1:1 dilution of Folin-Ciocalteu reagent was added and test tube was allowed to stand for a minimum of 30 mins at room temperature. The absorbance against blank (0 µL of the standard gallic acid standard solution) was determined at 750 nm using Thermo Scientific Varioskan Flash multimode plate reader. Gallic acid with concentrations 25, 50, 100, 200 and 400 µg/mL were used as standard for determination of total phenolic compounds in plant extracts. Total phenolic content was expressed as mg gallic acid equivalents per g of sample.[20,21]

Total Flavonoid Content

100 µL of stock extract was placed in a test tube. 100 µL of 2% (w/v) aluminium trichloride ($AlCl_3$) was added to test tube and incubated for 10 mins in the dark at room temperature. The absorbance against reagent blank (0 µL of the standard quercetin standard solution) was determined at 415 nm using Thermo Scientific Varioskan Flash multimode plate reader. Quercetin with concentrations 25, 50, 100, 200 and 400 µg/mL were used as standard for determination of total flavonoid content in plant extracts. Total flavonoid content was expressed as mg quercetin equivalents per g of sample.[22]

Table 1: Total phenolic and flavonoid contents of methanolic extract in three edible plants of Zingiberaceae family

Edibe Plant	Total phenolics (mg GAE/g sample)	Total falvonoid (mg QE/g sample)
Z. officinale	15.76 ± 0.003	111.68 ± 0.004
B. routinda	30.11 ± 0.002	42.63 ± 0.002
P. imperialis	42.65 ± 0.002	67.8 ± 0.007

Values are presented in mean ± SD (n=3). Total phenolics was expressed as mg gallic acid equivalent (mg GAE) in 1 g of dry sample and total flavonoid was expressed as mg quercetin equivalent (mg QE) in 1 g of dry sample.

DPPH(2,2-diphenyl-1-pycryl-hydrazyl) Radical Scavenging Assay

0.5 mL of plant methanolic extract was added to 0.2 mL of 0.1 mM ethanolic DPPH solution. The mixture was left to react in the dark at room temperature for 30 mins and measured spectrophotometrically at 518 nm.[23] DPPH radical scavenging activity (%) was calculated using the following formula:

[1-(Absorbance of sample-Absorbance of blank sample)/(Absorbance of control sample)]×100 where blank sample contained 0.2 mL of ethanol + 0.5 mL of sample/standards as positive control and control sample contained 0.2 mL of 0.1 mM DPPH solution + 0.5 mL ethanol.[24]

Cupric Ion Reducing Antioxidant Capacity (CUPRAC) Antioxidant Assay

$CuCl_2$ solution (1.0×10^{-2} M) was prepared by dissolving 85.24 mg of $CuCl_2.2H_2O$ in 50 mL H_2O. Ammonium acetate buffer (pH 7.0, 1.0 M) was prepared by dissolving 3.854 g NH_4Ac in 50 mL H_2O. Neocuproine (Nc) (7.5×10^{-3} M) was prepared by dissolving 78 mg of Nc in 50 mL of 96% EtOH. Samples were prepared to a final concentration of 50 µg/mL in H_2O. The procedure highlighted by Apak *et al*. was summarized as follows:

Add 1 mL 10^{-2} M Cu^{2+} + 1 mL 7.5×10^{-3} M neocuproine + 1 mL 1 M NH_4Ac + x mL antioxidant solution + (1.1 – x) mL H_2O; final volume 4.1 mL.[17]

The mixture was incubated at room temperature for 30 mins and read spectrophotometrically at 450 nm against a reagent blank.

Cellular Antioxidant Activity (CAA) Assay

CAA of plant methanolic extracts was measured using OxiSelect Cellular Antioxidant Activity (CAA) Assay Kit (Cell Biolabs, Inc.) in nasopharyngeal carcinoma cell line (HK-1). 2×10^4 HK-1 cells were seeded in a clear bottom black 96-well plate for 24 hrs. All media was removed and washed gently with phosphate buffered saline (PBS) for 3 times. 50 µL of 2',7'-dichlorodihydrofluorescin diacetate (DCFH-DA) probe was added to all wells before treated with 50 µL of plant methanolic extracts at different concentrations 31.3, 62.5, 125, 250, 500, 1000 and 2000 µM.

Plate was incubated for 60 mins. After incubation, all solutions were removed and wells were washed three times with PBS. 100 µL of free radical initiator solution was added to each well. Fluorescence was read at 37°C with excitation wavelength 480 nm and emission wavelength 530 nm for 60 mins with 5 mins intervals.

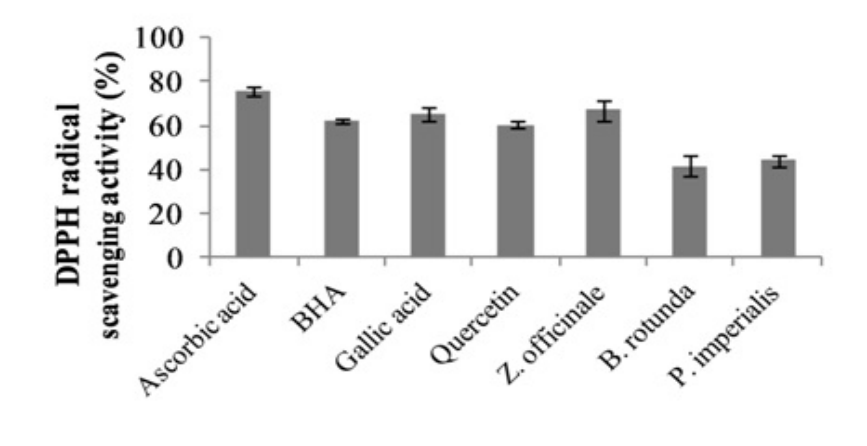

Figure 1: DPPH radical scavenging activity of *Z. officinale*, *B. rotunda*, *P. imperialis* and antioxidant standards.

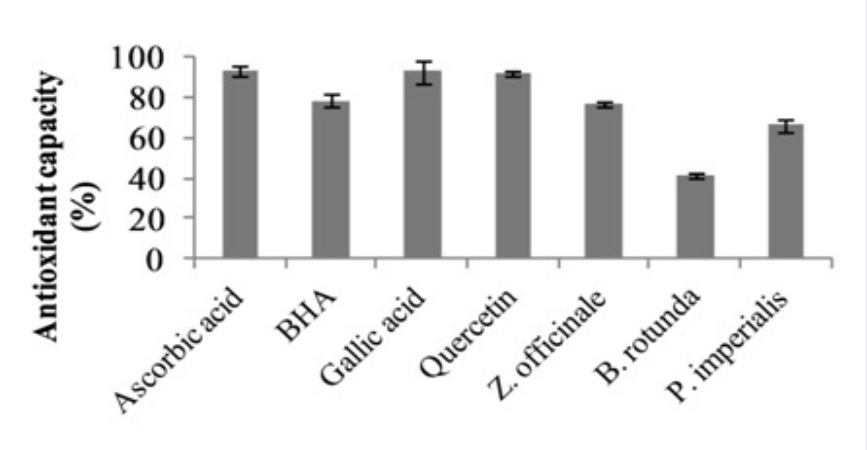

Figure 2: CUPRAC antioxidant capacity of *Z. officinale*, *B. rotunda*, *P. imperialis* and standard antioxidants.

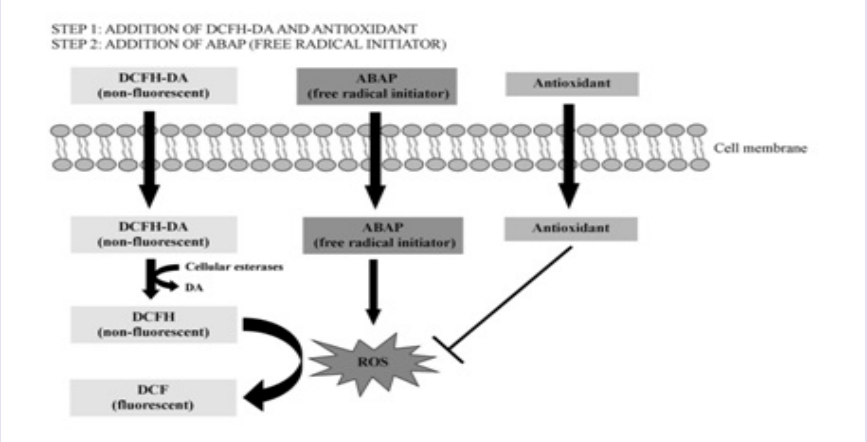

Figure 3: Schematic diagram illustrates steps in Cellular Antioxidant Activity (CAA) assay.

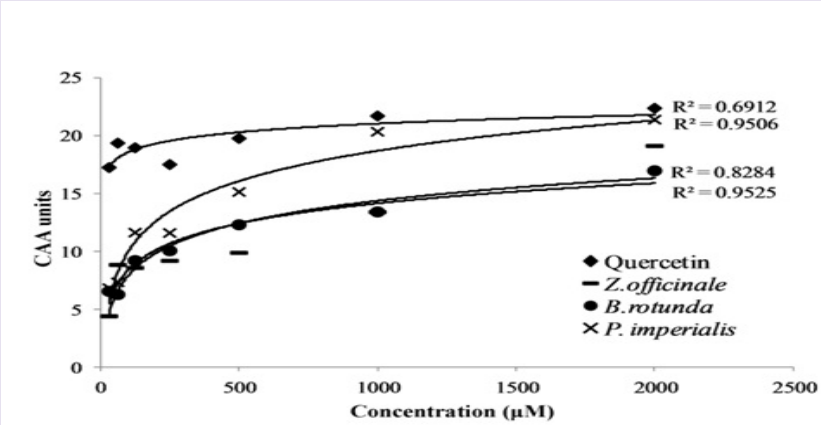

Figure 4: Dose-response curve of cellular antioxidant activity (CAA) of *Z. officinale*, *B. rotunda*, *P. imperialis* and quercetin antioxidant standard.

RESULTS

Total Phenolic and Flavonoid Content

Phenolics and flavonoids contents of three edible plants are presented in Table 1. *P. imperialis* has the highest phenol content, followed by *B. rotunda* and *Z. officinale*. On the contrary, *Z. officinale* contained most flavonoids, followed by *P. imperialis* and *B. rotunda*. It can be deduced that flavonoids present in *Z. officinale* are 7-fold the amount of phenolics. High flavonoids content present in *Z. officinale* methanolic extract compared to other edible plants may further cement a promising anti-oxidant property.

DPPH (2,2-diphenyl-1-pycryl-hydrazyl) Radical Scavenging Assay

This is a very common method to determine radical scavenging capabilities. DPPH itself is a radical and, in ethanol solution, has a deep violet colour, with a strong peak at approximately 520 nm. When DPPH is mixed with a reducing agent, i.e. a substance that can donate a hydrogen atom, it becomes the reduced form of DPPH. The violet colour is replaced with a dull yellow colour and has a lower absorption at 520 nm.[23]

The DPPH radical scavenging activity of the edible plant methanolic extracts and standard antioxidants increased in the order of *B. rotunda*<*P. imperialis*< quercetin < BHA < gallic acid <*Z. officinale* < ascorbic acid (Figure 1).

Cupric Ion Reducing Antioxidant Capacity (CUPRAC) Antioxidant Assay

CUPRAC assay was employed to determine reducing power of edible plant extracts and standard antioxidants. The reaction was based on the reduction of Cu^{2+}-Cu^{+} ions by antioxidant in the presence of neo-cuproine. It has been previously reported that CUPRAC has effectively demonstrated various advantages over other antioxidant assays. It is relatively more sensitive and environment where reaction occurs is near to physiological pH.[15] In present findings, reducing power decreased in the order of gallic acid > ascorbic acid > quercetin > BHA >*Z. officinale*>*P. imperialis*>*B. rotunda* (Figure 2). All there methanolic edible plant extracts showed lower reducing capability than standard antioxidants. However, antioxidant capacity of *Z. officinale* methanolic extract was shown to be highest among all plant extracts.

Cellular Antioxidant Activity (CAA) Assay

This method involves a fluorogenic probe dye 2',7'-dichlorohydrofluorescin diacetate (DCFA-DA) that is able to diffuse into cells (Figure 3). Esterases in cells deacetylate DCFA-DA to non-flourescent 2',7'-dichlorodihydrofluorescin (DCFH). HK-1 cells were pre-incubated with cell-permeable DCFA-DA fluorescence probe and quercetin (antioxidant standard) or methanolic plant extracts.

2,2'-Azo-bis-amidinopropane (ABAP), a free radical initiator causes generation of peroxyl radicals which leads to a rapid oxidation of DCFH to highly fluorescent 2'7'-dichlorodihydrofluorescein (DCF). Antioxidant present in plant extracts will quench these peroxyl radicals by preventing the generation of DCF. Therefore, CAA assay measures the ability of antioxidants to inhibit oxidation of DCFH to DCF. The conversion from non-fluorescent DCFH to fluorescent DCF act as an oxidative stress indicator. Quercetin was employed as standard for CAA assay because it is pure, relatively stable and widely found in fruits and vegetables.[18]

Current finding showed that cellular antioxidant activity (CAA) of the edible plant methanolic extracts and quercetin standard antioxidant decreased in the order of quercetin >*P. imperialis* >*Z. officinale* >*B. rotunda* (Figure 4).

DISCUSSIONS

Edible plants have been a great source for drug development. Edible plants are safe, easily available and possess tremendous medicinal values. Edible plants made into herbs have been proven to not only possess antioxidant ability but also display anticancer properties.[25] Plants belonging to the Zingiberaceae family have long been used as folklore medicine to treat various ailments. In current research, we have selected three commonly edible plants from this family in search of potential free radical scavengers. Edible plants were macerated with methanol solvent to extract all polar compounds which may potentially exert antioxidative properties. Phenolic and flavonoid contents were evaluated for all methanolic extracts.

In current research, *Z. officinale* methanolic extract displayed higher radical scavenging activity than two other plant extracts. Both phenols and flavonoids are bioactive compounds present in most plants and possess robust antioxidant activity. A significant finding reported that increasing phenol content leads to an increased antioxidant activity.[26-28]

However, total phenolic content in *Z. officinale* methanol extract was relatively low. Therefore, it can be deduced that the antioxidant action may be due to the action of flavonoid and not phenolic compounds present in the plant extract. The actions of radical scavenging may be contributed by high flavonoids present in *Z. officinale* methanolic extract.

Recent research suggested the association between both flavonoids and phenols content and antioxidant activity. It is likely that the plant with the highest concentration of one or both moieties will exhibit most potent antioxidant activity.[11]

CAA assay is greatly dependent on the properties of antioxidants and their interactions with cells. Antioxidant can react by inhibiting peroxyl radical chain on cell surface or react intracellularly. The structural properties of flavonoids and other phytochemicals such as polarity also determine the interactions on the cell membrane.[29] It has been proven previously that flavonoid such as quercetin contains 2,3-double bond and 4-oxo group displayed high CAA.[18] Methanolic extract of *P. imperialis* contains highest phenols content and highest CAA compared to *Z. officinale* and *B. rotunda*. CAA values were reported to be significantly correlated with total phenolics content in common fruits.[30] Current result suggests that phenolics content in *P. imperialis* may contribute to CAA in nasopharyngeal carcinoma, cells.

CONCLUSION

From this study, we can conclude that methanolic extract of *Z. officinale* revealed highest flavonoid content and displayed most effective anti-oxidant capability in DPPH and CUPRAC antioxidant assays compared to *B. rotunda* and *P. imperialis* in Zingiberaceae family. Methanolic extract of *P. imperialis* contains highest phenols content which associated with high antioxidant activity (CAA assay). Future work involves elucidation of the compound responsible to trigger the actions of radical scavenging.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

ABBREVIATION USED

DPPH: 2,2-diphenyl-1-picryl-hydrazyl; **CUPRAC:** Cupric ion reducing antioxidant capacity; **CAA:** Cellular antioxidant activity.

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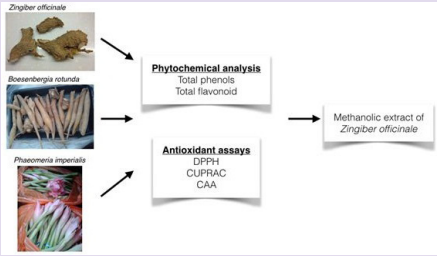
PICTORIAL ABSTRACT



SUMMARY

- Methanolic extract of Z. offici-nale displayed highest free radical scaveng-ing ability.
- *Revolutionary CUPRAC and CAA *in vitro* antioxidant assays employed were able to accurate-ly evaluate the degree of radical scavenging capa-bility in edible plants.
- *There was a strong correlation between the presence of flavonoids in Z. officinale and its anti-oxidant activity.

ABOUT AUTHOR

Teng-Jin Khoo: Is a Associate Professor at The University of Nottingham Malaysia campus. He obtained his phD in 2007 major in Synthesis and Organome-tallic Chemistry. He is currently working on various aspects of semi-synthesis of natural com-pounds and drug discovery.