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Research Article

Identification of Antioxidant Activity of Medicinal Plant Extracts Through a Modified and Rapid ABTS Colorimetric Assay

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Abstract

The consumption of antioxidant rich plants is a common human activity. Therefore, easily used, time efficient and effective antioxidant assays of plants consumed by humans are of increasing importance. The ABTS [2,2'-azinobis-(3-ethtlbenzothiazoline-6-sulfonic acid)] cation radical is commonly used in colorimetric assays to assess antioxidant properties of test compounds. Oxidation of ABTS (7.0 mM) to the ABTS radical is often performed by overnight (6-12 h) reaction with 2.45 mM potassium persulfate. This was subsequently followed by dilution of the radical to an appropriate working absorbance at 734 nm. Described in the current study is the incorporation of 0.7 mM ATBS (rather than the previously described 7.0 mM) that rapidly (30 min) goes to complete oxidation and formation of the ABTS cation radical. This rapidly provided a suitable stable radical for antioxidant studies, thus circumventing the need for overnight (6-12 h) incubation. This protocol was subsequently incorporated into a microtiter (high-throughput) plate format. Thus, multiple dilutions and determinations of antioxidant activity of test compounds could be determined in less than one hour, which included time for formation of the ABTS radical. This protocol was successfully used to determine antioxidant activity in plant extracts that are widely used dietary supplements and consumed most often in the form of tea. Results showed that antioxidant activity of certain plant extracts at higher concentrations (125 µg/ml) was comparable to a Trolox standard. Plant extract antioxidant activity rapidly diminished in a stepwise manner following twofold serial dilutions in the microtiter plate format. Trolox antioxidant activity diminished in a stepwise fashion at much higher dilutions. The spectrophotometric absorbance readings were highly reproducible with a characteristically low standard deviation. Application of this rapid colorimetric assay in the identification of antioxidant properties has notable results in these traditionally used plants along with potential for use in future studies of plant constituents.

Keywords:
ABTS assay; rapid high throughput; plant extracts; Cyclocarya paliurus; Gynostemma pentaphyllum; Usnea diffract

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Introduction

Antioxidant-rich compounds are a feature of a variety of plants that can be consumed by humans. Thus, the consumption of certain plants has the potential to be of benefit to humans [1,2] Assessment of antioxidant activity in foods, teas, and natural products used for their purported benefit to humans continues to an active pursuit in the scientific community. The ABTS assay has gained acceptance as a valuable tool in the assessment of antioxidant activity and pursuit of these studies. The ABTS colored cation radical is generated upon the loss of an electron by the nitrogen atom of ABTS [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)] [2]. For antioxidant assay purposes, this reaction (oxidation and loss of an electron and formation of the ABTS cation radical) can be brought about by use of potassium persulfate ($K_2S_2O_8$) [3]. With use of potassium persulfate, the ABTS radical was generated directly with no intermediary radical. Thus, the ABTS radical is pre-formed prior to addition of test antioxidants, eliminating the possibility that test substances could interfere with generation of the ABTS radical [3]. Use of potassium persulfate for generation of ABTS radical has garnered popularity [3,4,5,6]. Spectrophotometer readings at absorbance setting of 734 nm were regularly used and Trolox has often served as an antioxidant standard [3,4,5,6].

A feature of this assay system has been the use of 2.45 mM potassium persulfate in conjunction of 7.0 mM of ABTS for ABTS radical generation. This has been accompanied by overnight (12-16 h) incubation and subsequent dilution of the ABTS radical is to a working absorbance value of approximately 0.7 (or slighter higher in some studies) at 734 nm [3,4,5,6]. Working assay volumes were typically 1.0 ml (or greater in some studies). Absorbance and antioxidant activity is most often determined within the first 6 min after addition of antioxidant [3,4,5].

Described in this current study is the use of 0.7 mM ABTS (rather than the previously described use of 7.0 mM ABTS) in conjunction with 2.45 mM potassium persulfate. When this scheme was followed, the generation of the ABTS radical went to completion in 30 min thus avoiding the previously described overnight (12-16 h) incubation period. The resulting ABTS radical was stable and amenable to incorporation to a microtiter plate format which is also described in this study.

This format afforded the opportunity to initiate and assess antioxidant activity in less than one hour, which included generation time for radical formation and assessment of test antioxidants. The mi-

crotiter plate format afforded the ability to more easily perform serial dilutions and multiple determinations (triplicate) of test antioxidant compounds. An additional benefit of this modified assay is the reduced amount of test reagents needed to access antioxidant activity as compared to the use of ml test volumes previously described [3,4,5,6].

Increasing popularity of dietary supplements consumed for their proposed health benefits is an increasing practice which often entails antioxidant testing for validation of claims of antioxidant activity. This format served as a practical means to rapidly assess antioxidant activity in extracts of plants that are popular diet supplements consumed in the form of tea, especially in China. Test results demonstrated antioxidant activity and variability among the different plant extracts. Multiple determinations of antioxidant activity of serial dilutions of plant extracts based on this format were consistent and reproducible. This modified and more rapid ABTS colorimetric assay for determination of antioxidant activity also has potential applications for assessment of antioxidant activity in a variety of other biological specimens.

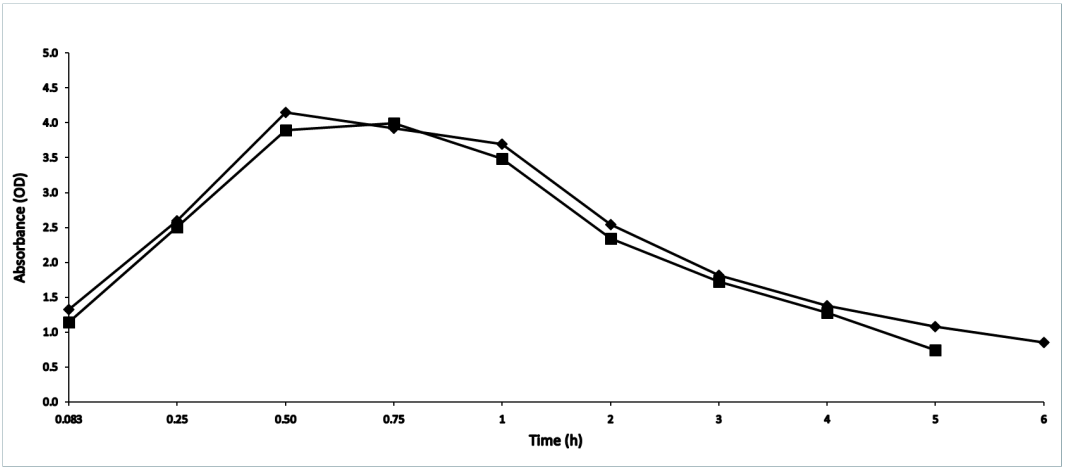


Figure 1. Conversion of 0.7mM ABTS to the ABTS cation radical with 2.45 mM potassium persulfate.

Values represent average of triplicate determinations. The SD was consistently less than 0.11. Deionized Water (■), PBS (◆).

2. Materials and Methods

2.1. ABTS radical preparation protocol

ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) was dissolved in 1X phosphate buffered saline (HyClone Laboratories, Logan UT). For this, 5mg ABTS (Roche) tablets (Sigma-Aldrich, St. Louis, MO) were added to 7 ml PBS which yielded a final concentration of 0.7 mM ABTS. A 2.45 mM solution of potassium persulfate (Sigma-Aldrich, St. Louis, MO) was prepared in deionized water. A volume of 7.0 ml of ABTS was added to 7.0 ml of potassium persulfate in a glass tube and placed in the dark at room temp (22 ºC). The starting ABTS solution was prepared fresh before use and the potassium persulfate prepared fresh every 2 weeks. Absorbance readings showed that conversion of ABTS to the ABTS radical went to completion in 30 m (Fig 1). The newly formed ABTS radical was suitably stable for immediate dilution in PBS and use in antioxidant studies.

2.2 Microtiter plate preparation

While the ABTS radical formation was in progress (30 min), test microtiter (96-well polystyrene clear flat bottom) plates (Denville Scientific Inc., Charlotte, NC) were prepared. For this, 50 µl of PBS was added to all wells except for the outside row of wells on each side of the microtiter plate. A volume of 50 µl of test compound was added to the first test well and the 50 µl removed and serially diluted across the row and the final 50 µl from the last test well in the row was discarded. A multichannel pipette (VistaLab Technologies, Brewster, NY) was used to perform tests in duplicate, triplicate, or whatever number of multiple repeats were deemed appropriate. The number of replicates

(triplicate replicates) in a typical assay along with the standard deviation (SD) appears in each figure legend. The assay test outcomes were independently reproducible within the laboratory setting over additional timepoints throughout the study period.

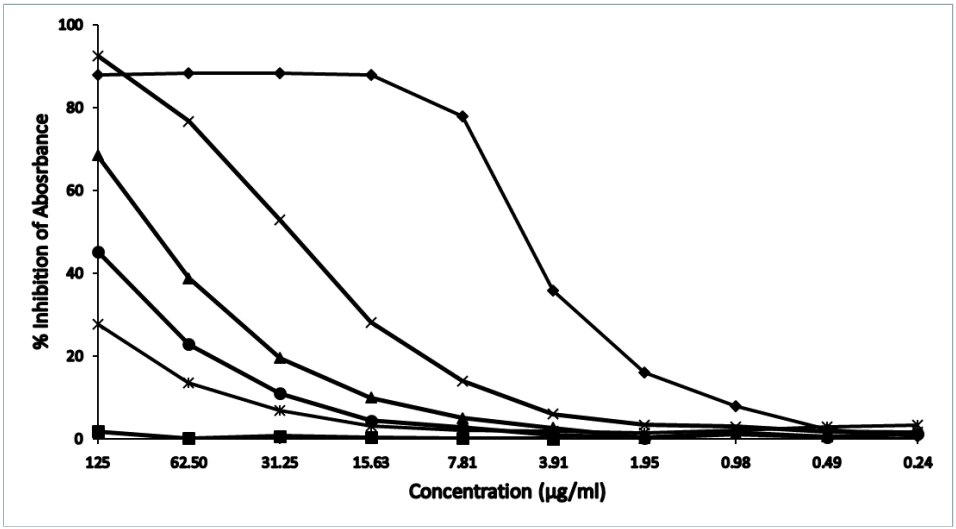


Figure 2. Percent inhibition of absorbance (after 5 min) of ABTS cation radical from extracts from *Cyclocarya paliurus*.

Values represent average of triplicate determinations. The SD was consistently less than 0.084. Petroleum ether (✱), Ethyl acetate (▲), Butanol (X), Chloroform (●), Trolox (◆), DMSO (∎).

2.3 Test compounds

Plant extracts were provided by Guangxi Botanical Garden of Medicinal Plants (Nanning, PR China). The solvents petroleum ether, ethyl acetate, butanol, or chloroform were used for preparation of extracts. Preparations were taken to dryness and subsequently suspended in dimethyl sulfoxide (DMSO) at a concentration of 10 mg/ml. DMSO only served as a negative control for the antioxidant studies. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (Sigma-Aldrich, St. Louis, MO) served as an antioxidant standard and was likewise suspended in DMSO at 10 mg/ml.

2.4 Test assay

Once dilutions of test compounds were prepared on the microtiter plates, 150 µl of the ABTS radical was added to each well (total volume 200 µl/well) with a multichannel pipette to give an initial absorbance of 0.7 at 734 nm (CLARIOstar Microplate Reader, BMG Labtech Inc., Cary, NC). Absorbance readings (at 734 nm) were determined at room temp at 5, 15, and 30 min after addition of the ABTS radical to test compound dilutions. Antioxidant activity of test compounds, Trolox, and DMSO reflected percent inhibition of absorbance where percent inhibition of absorbance = 100 – (average extract absorbance x 100) / average DMSO only absorbance.

3. Results

Incorporation of 0.7 mM (rather than the previously described use of 7.0 mM) ABTS resulted in oxidation to completion of ABTS to the ABTS cation radical in 30 min as noted by absorbance at 734 nm (Fig1). The radical was suitably stable for the assessment of antioxidant activity of test compounds. Assessment was performed immediately after oxidation of ABTS and complete formation of the ABTS radical.

Antioxidant activity (percent inhibition of absorbance of ABTS radical) of plant extracts and the Trolox standard was apparent after 5 min. (Figure 2, Figure 3, Figure 4). Additional measurements of antioxidant activity of up to 30 min did not yield substantial differences from that observed at 5 min (data not shown). Thus, figures represent antioxidant activity observed at the 5 min time point. Antioxidant activity of plant extracts varied among the plants tested and the plant extraction methods. The DMSO only microtiter test wells displayed negligible antioxidant activity.

Antioxidant activity of extracts from *Cyclocarya paliurus* was apparent after 5 min (Fig 2). All extracts displayed antioxidant activity at the highest concentration tested (125 µg/ml). The butanol extract displayed the greatest antioxidant activity and was equivalent to that of Trolox at the highest concentration (125 µg/ml). Its antioxidative activity remained greater than that of the other extracts (petroleum ether, ethyl acetate, and chloroform) per dilution to lower concentrations on the microtiter plate. Following a 2-fold dilution to 62.50 µg/ml in the microtiter plate well only the butanol extract displayed greater than 40 percent inhibition of absorbance. The Trolox standard displayed a 40 percent or greater inhibition of absorbance to a concentration of approximately 3.91 µg/ml.

Antioxidant activity of extracts of *Gynostemma pentaphyllum* was likewise demonstrated (Fig 3). Greatest antioxidant activity was associated with the ethyl acetate extract. Even at the highest concentration tested (125 µg/ml), inhibition of absorbance did not exceed 60 percent. Less than 40 percent inhibition was observed in all the *G. pentaphyllum* extracts at 62.50 µg/ml or less (Fig 3). A characteristic of the antioxidant activity of extracts from *Usnea diffracta* was that the greatest antioxidant activity was again associated with the butanol extract (Fig 4). At its highest concentration (125 µg/ml) it was equivalent to that of Trolox. Following a 2-fold dilution to 62.50 µg/ml in the microtiter plate well greater than 40 percent inhibition of absorbance was still apparent in the butanol and chloroform extracts.

A feature of the antioxidant activity of all the plant extracts compared to Trolox was the rapid diminishment of antioxidant activity of plant extracts following 2-fold dilutions on the microtiter plates. Trolox antioxidant activity continued to be apparent following the 2-fold serial dilutions and a nearly 40 per cent inhibition of absorbance was displayed by Trolox at a concentration of 3.91 µg/ml. Antioxidant activity was not associated with DMSO alone control microtiter plate wells.

4. Discussion

The newly described format functioned effectively for assessment of antioxidant activity in plant extracts that are used and consumed by humans. The primary application of *Cylocarya paliurus* has been use of its leaves in tea production and antioxidant properties have been ascribed to water soluble extracts of the leaves. Among 21 different geographic populations of the plant, there was a significant difference in antioxidant activities [10 Bith *Gynostemma pentaphyllum* and *Usnea diffracta* components are reportedly used in human activities [11,12]. Antioxidant activity has been demonstrated in additional *Usnea* species [13].

In the current study, the greatest level of antioxidant activity in two of the three plant extracts was associated with extraction by butanol. At the highest concentration tested (125 µg/ml) antioxidant activity was comparable to that of a Trolox standard (also at 125 µg/ml). The antioxidant activity of the plant extracts rapidly diminished upon dilution in the microtiter plate format. The Trolox standard, however, maintained prominent antioxidant upon dilution to much lower concentrations (µg/ml) of antioxidant.

Generation of the ABTS radical using potassium persulfate is a conventional approach for assessment of antioxidant activity. The ABTS and potassium persulfate react in a stoichiometric manner yielding the ABTS cation radical. Previously this approach has carried with it a requirement to allow at least 6 h, and typically a 6 to 12 h overnight reaction for oxidation completion formation of the stable ABTS radical cation for antioxidant studies [3,4,5,6].

In the current study, the capability to generate a suitably stable ABTS cation radical using potassium persulfate in 30 minutes for antioxidant studies provides a logistic alternative to the previously described 6 h minimum reaction time [3,4,5,6]. Furthermore, the ABTS radical was readily adaptable into a microtiter-based format providing an additional advantage of easily performed repetitive testing of different dilutions of test antioxidant.

Assessment of antioxidant activity in nutritional products and especially plants used and consumed by humans continues to be an important scientific activity. With the continued scientific interest in assessment of antioxidant activities, there also continues to be efforts directed at efficient and cost-effective protocols for assessment of antioxidant activities and high-throughput microtiter-based assays have been incorporated into antioxidant assays [7,8,9].

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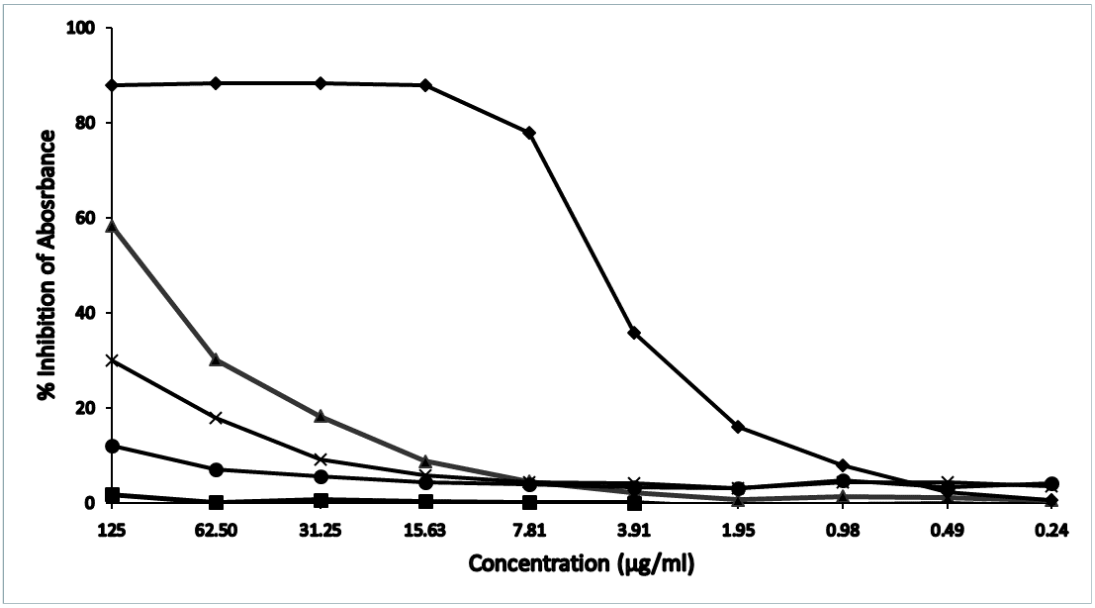


Figure 3. Percent inhibition of absorbance (after 5 min) of ABTS cation radical from extracts from *Gynostemma pentaphyllum.* Values represent average of triplicate determinations.

The SD was consistently less than 0.092. Ethyl acetate (▲), Butanol (X), Chloroform (●), Trolox (◆), DMSO (∎).

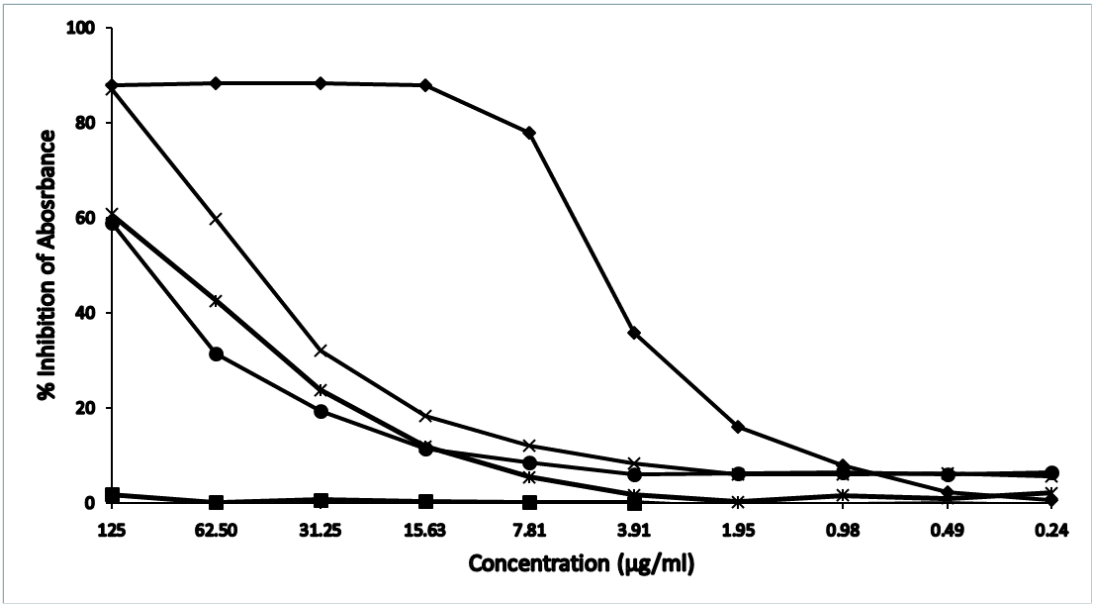


Figure 4. Percent inhibition of absorbance (after 5 min) of ABTS cation radical from extracts from *Usnea diffracta*.

Values represent average of triplicate determinations. The SD was consistently less than 0.018. Petroleum ether (✱), Butanol (X), Chloroform (●), Trolox (◆), DMSO (∎).

Conclusion

Collectively, the results of this study provide a much more rapid (1 h) alternative for determinations of antioxidant activity using the ABTS/potassium persulfate-based format. The incorporation of a microtiter-based format additionally supports rapid assessment of multiple determinations of test dilutions of antioxidant compounds. In addition to use in dietary plant extracts as described above, this accelerated method also provides potential for expediting the evaluation of antioxidant and anti-inflammatory properties for a variety of studies involving other plants consumed by humans and products derived from plants for human use.

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Competing Interests

The authors have declared that no competing interests exist.

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