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SHORT COMMUNICATION

Can MTT be used to quantify the antioxidant activity of plant extracts?

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Abstract

Antioxidant properties of three different medicinal plant extracts from northern Nigeria were evaluated using the traditional qualitative 2,2, diphenyl-picrylhydrazyl (DPPH) method and a new 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) microdilution method described here. The results were in agreement. It is a simple, rapid and inexpensive method compared with many other methods for determining quantitative antioxidant activity of plant extracts.

Keywords: Antioxidant; Plant extracts; DPPH; MTT

Introduction

Antioxidant compounds play an important role in preventing or delaying the onset of major degenerative diseases (Mazza, 1998). The physiological role of antioxidant compounds is to scavenge for free radicals (Surai, 2002). Active oxygen (hydroxyl, peroxyl radicals and singlet oxygen) is highly toxic and an important causative agent of many diseases including cancer, heart disease, cataract and congestive disorders. Antioxidant compounds block the oxidation processes that produce free radicals which contribute towards these chronic diseases and aging (Ames, 1983; Block, 1992). Free radicals are highly unstable and reactive species are capable of damaging molecules such as DNA, proteins and carbohydrates. The body is under constant attack from these free radicals formed as a consequence of the body's normal metabolic activities (Surai, 2002). Antioxidants therefore play an important role in animal health. Conventional antioxidants improve animal performance during conditions characterized by increased tissue oxidant levels such as stress, injury and infection (Nockel, 1996).

Vitamin E is an excellent biological free radical chain-breaking antioxidant that protects cells and tissues from lipoperoxidative damage induced by free radicals (McDowell et al., 1996). Vitamin C also plays an important role in animal health as an antioxidant supplement by inactivating free radicals produced through normal cellular activity and diverse stressors (Halver, 1995).

Plant-derived antioxidants exert their effects by enhancing the levels of antioxidant enzymes such as superoxide dismutase and glutathione peroxidase or by lowering the levels of lipid peroxides in the blood or liver (Usoh et al., 2005; Tseng et al., 1997). It is recognized that antioxidant (mainly polyphenolic) compounds from plant extracts can act by either free radical scavenging (Lodovici et al., 2001; Re et al., 1999), singlet oxygen quenching (Foley et al., 1999), chelating of transitional metal such as iron (Brown et al., 1998), as well as a reducing agents and activator of antioxidative defense enzyme systems to suppress radical damage in biological system (Zielinski and Kozlowska, 2000).

Recently there has been an increased concern about synthetic antioxidants. This is partly due to their

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possible toxicity against animal DNA. This may explain the interest in examining plant extracts as a source of cheaper and effective antioxidants and the growing interest in nutraceuticals.

There is no single, widely acceptable assay method for antioxidant capacity applicable to reasonable variety of compounds and matrices (Ozyurt et al., 2007) but the most commonly used methods for measuring activity are those that involve the generation of a free radical species which are then neutralized by antioxidant compounds (Arno et al., 2001). Qualitative 2,2, diphenyl-picrylhydrazyl (DPPH) method as well as quantitative trolox equivalent antioxidant capacity (TEAC) method have been frequently used for antioxidant determination.

In determining the antibacterial activity of different plant extracts using a serial microplate dilution method (Eloff, 1998) there were difficulties in using tetrazolium violet as indicator of growth due to the colour of some of the extracts. When we used 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) we noticed in the negative controls that MTT was changed to the purple formazan by some plant extracts without the bacteria. This then drew our attentions for further investigations.

In this study we report for the first time the use of MTT in a microtitre plate to quantify the scavenging ability of the antioxidant compounds present in three different plant extracts. We also compare the results obtained with the qualitative DPPH method.

Materials and methods

The plant materials used in this experiment (i.e. *Terminalia laxiflora* Engl. & Diels, *Allium sativum* L. and *Agava sisalana* Perrine) were collected from some parts of northern Nigeria between February and March 2007. The leaves were sun-dried and pulverized. The powdered plant materials were extracted with acetone at a ratio of 1:15 m/v in the Phytomedicine Laboratory of University of Pretoria South Africa (www.up.ac.za/phyto).

To evaluate the number of antioxidant compounds present in an extract, 10 µl of 10 mg/ml of each plant extract was spotted on a TLC plate and eluted in a polar solvent comprising the mixture of ethyl acetate, methanol and water in the ratio of 10:1.35:1 (Kotze and Eloff, 2002). The developed plate was dried and immediately sprayed with 0.2% DPPH in methanol (Sigma).

Similarly, in an alternative MTT assay, a two-fold dilution of 10 mg/ml of each extract was carried out with 100 µl of distilled water in a 96-well microdilution plate. Thereafter, 50 µl of 0.2 mg/ml of MTT was added to every well and the plate was incubated for 2–3 h at 37 °C after which the result was read. The lowest concentration of the extract at which the presence of antioxidant is detected was recorded as the highest dilution at which the formation of bluish-purple colouration disappears. This experiment was done in triplicate and was repeated over a period of time to evaluate the consistency of result.

Result and discussion

The extract of *T. laxiflora* at 10 mg/ml (100 µg) had a better antioxidant compound(s) on 0.2% DPPH-sprayed chromatograms. This was indicated by the appearance of a long yellow band on a purple background but the extract of *A. sativum* barely showed the presence of the band at the same concentration signifying a very low detection. The chromatogram of the extract of *A. sisalana* did not indicate the presence of antioxidant compound at that concentration as there was no detection of the band.

The result of MTT was comparable with that obtained with the DPPH method. The bluish-purple formazan in *T. laxiflora* was observed from the first dilution at 5 mg/ml up to eight dilutions at 0.04 mg/ml in a 2-fold dilutions signifying presence of antioxidant compound at those concentrations. There was no such colour formation with the extract of *A. sisalana* even at the highest concentration of 5 mg/ml denoting absence of antioxidant activity at that concentration. Similarly, the extract of *A. sativum* showed colour formation from the first dilution at 5 mg/ml up to fifth dilutions at 0.31 mg/ml. This showed the lowest concentration of the extract to which antioxidant activity can be detected. Since the DPPH method only signifies the presence of antioxidant, the total amount used for the detection was 100 µg (10 µl of 10 mg/ml) for the three plants and the results showed a better detection with a good band in *T. laxiflora* followed by *A. sativum* with a nip of the band, and a poor detection by *A. sisalana* which has no band. The MTT method which involves a serial dilution required a total amount of 1000 µg (100 µl of 10 mg/ml) for each plant for ease of dilution and the detection was at 40, 310 and above 5000 µg for *T. laxiflora, A. sativum* and *A. sisalana*, respectively, which is in accordance with the DPPH method.

The qualitative analysis of antioxidant activity of plant extracts using the DPPH assay on thin layer chromatographic (TLC) plate is a simple method used as a screening test for the radical scavenging ability of the antioxidant compounds present in plant extract (Sanchez-Moreno et al., 1998). DPPH is a purple coloured compound which is a stable free radical that on contact with the antioxidant, it either transfers electrons or hydrogen atoms to it thereby neutralizing its free radical character (Naik et al., 2003). The quantitative DPPH spectrophotometric analysis of antioxidant activity is more complicated and we have applied it in several studies (Aderogba et al., 2007).

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We have also used the quantitative TEAC assay that involves prior generation of the radical monocation 2,2,-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid; $ABTS^+$). The blue/green chromophores $ABTS^+$ is produced through the reaction between ABTS and potassium sulphate (Re et al., 1999). The addition of antioxidants to the free radical reduces it to a colourless $ABTS^+$, a reaction that depends on the concentration of the antioxidant and the duration of the reaction. We experienced several problems with the reproducibility of the data when applied to plant extracts.

There is a need for developing new methods of determining antioxidant activity of extracts. Because of the high cost and relatively unstable radical chromogen reagents such as ABTS and DPPH, an easily adaptable and low-cost method known as cupric ion reducing antioxidant capacity (CUPRAC) was developed for total antioxidant assay in food stuff and human plasma (Apak et al., 2004, 2005). Recently, an indirect spectrophotometric method that is more accurate, simple, low-cost, sensitive and inexpensive has also been developed for the determination of total antioxidant capacity of simple antioxidant compounds of several plants (Ozyurt et al., 2007). This method is based on the oxidation of antioxidant compound with cerium (IV) sulphate at room temperature under controlled conditions of reagent concentrations and pH. It is the first documentation of Ce (IV)-based total antioxidant assay for a wide variety of antioxidant compounds individually and in mixtures and is known as cerium ion reducing antioxidant capacity (CERAC).

In conclusion, the MTT method used in the present study for assaying of antioxidant capacity which is based on reduction of MTT by antioxidant compound is also a simple, rapid, inexpensive, sensitive and accurate method. Because MTT is used so widely in cell culture work, this may be a useful technique. The method appears to be reproducible and requires little material for detection and quantification of antioxidant activity in plant extracts. The method can be employed as a first screening approach of plant extracts for antioxidant capacity before a detailed study of antioxidant mechanisms is conducted.

A more in-depth study is required to compare aspects such as sensitivity and reproducibility obtained with different plant extracts with different techniques. It may also be useful to evaluate the interaction between plant extracts, antioxidant compounds in these plant extracts and antibacterial activity determined by the serial microplate INT method (Eloff, 1998) to evaluate if antioxidant activity of extracts has an influence on the antibacterial activity measured by this technique.

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