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Critical analysis of current methods for assessing the *in vitro* antioxidant and antibacterial activity of plant extracts

Joash Ban Lee Tan *, Yau Yan Lim

School of Science, Monash University Malaysia, Bandar Sunway, 46150 Petaling Jaya, Selangor, Malaysia

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2,2′-Azobis-2-methyl-propanimidamide dihydrochloride (AAPH) (PubChem CID: 76344)
Fluorescein (PubChem CID: 16850)
Ferric tripyridyltriazine (PubChem CID: 77258)
p-Dimethylaminocinnamaldehyde (DMACA) (PubChem CID: 5284506)
$AlCl_3$ (PubChem CID: 24012)
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ABSTRACT

Natural product research is an active branch of science, driven by the increased value placed on individual health and well-being. Many naturally-occurring phytochemicals in plants, fruits and vegetables have been reported to exhibit antioxidant and antibacterial activity; often touted as being beneficial for human health. *In vitro* screening is a common practice in many research laboratories as a means of rapidly assessing these properties. However, the methods used by many are not necessarily optimal; a result of poor standardization, redundant assays and/or outdated methodology. This review primarily aims to give a better understanding in the selection of *in vitro* assays, with emphasis placed on some common assays such as the total phenolic content assay, free radical scavenging activity, disc-diffusion and broth microdilution. This includes a discussion on the reasons for choosing a particular assay, its strengths and weaknesses, ways to improve the accuracy of results and alternative assays.

1. Introduction

Oxidative stress has been a major research interest in the past few decades due to its implicated role in human diseases such as cancer, atherosclerosis and brain dysfunction (Patel, Kumar, Prasad, & Hemalatha, 2011). Plant secondary metabolites, or "phytochemicals", are produced by plants for a myriad of functions, from UV protection, protection against pathogens and herbivores, pigmentation to improve chances of pollination, and other means of improving the plant's survivability and health, without being directly involved in crucial functions like the growth and reproduction (Bagniewska-Zadworna, Zenkteler, Karolewski, & Zadworny, 2008). In the past few decades, there has been a great increase in scientific interest around these compounds and their benefit to human health, as many exhibit considerable antioxidant and antibacterial activity (Bansal et al., 2013; Cartea, Francisco, Soengas, & Velasco, 2011). This interest in natural antioxidants is further reinforced by the increasing doubt towards synthetic antioxidants, which may be carcinogenic and unstable (Chandra et al., 2014). Approximately 14–28% of higher plant species have an ethnobotanical application, and a majority of these active plant-derived compounds were identified following further research based on traditional medicinal uses (Ncube, Afolayan, & Okoh, 2008).

* Corresponding author. Tel.: +60 3 55146107.
E-mail address: tan.ban.lee@monash.edu (J.B.L. Tan).

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Given the importance and sheer volume of natural product research, it is crucial to understand the options, limitations, and potential improvements in the selection of assays used for the *in vitro* evaluation of antioxidant and antibacterial activity. The evaluation of plant bioactivity is complicated, as no single assay or combination of assays is necessarily optimal given the broad diversity of chemical compounds present (Power, Jakeman, & FitzGerald, 2013). Unfortunately, some of the work in this field is mired in a loop of routine methodology often using outdated assays and/or a combination of assays that only show an aspect of the plant's antioxidant or antibacterial activity which is then erroneously reported as the overall bioactivity.

A major issue is the lack of standardized methodology in *in vitro* assays, thus hindering comparisons (Power et al., 2013). While certain assays appear somewhat more commonly, even within the same methodology, results can be published in different forms or units that make cross-comparison between the literature sometimes impossible. Standardization of protocols is therefore crucial in ensuring comparability of results between different samples, authors and research groups. While not all variables can be easily standardized, chief amongst these being environmental factors between different geographical locations, many of the methodologies employed in the laboratory can be standardized, such as extraction protocols, choice of assays, assay conditions and units for reporting data (Ncube et al., 2008).

Rather than focusing on the complicated mechanisms behind each topic, this review aims to give a practical and critical overview of the options and factors to consider in choosing the correct antioxidant and antibacterial assays when planning a natural product-based project; highlighting strengths and weaknesses of different methods in the hope of ultimately improving the quality and comparability of reported work in this field.

2. Antioxidant activity

2.1. The basics of antioxidant evaluation

To best appreciate the information to follow, the basis of the assays used in *in vitro* antioxidant determination must be first understood. It is important to note that *in vitro* chemical assays bear no similarity to biological systems, and the absorption of antioxidant compounds by the human body (Bjelakovic, Nikolova, Gluud, Simonetti, & Gluud, 2007), has been a hotly debated topic in the past few decades. Consequently, all results generated by an assay are accurate only within the reaction conditions of that particular assay (pH, temperature, reactants, and so on). Hence, it would be inaccurate to broadly claim the bioactivity of a sample based purely on *in vitro* assays since none of them account for bioavailability, stability, retention or reactivity *in vivo* (Huang, Ou, & Prior, 2005).

Despite the controversy, *in vitro* screening for antioxidant activity and high antioxidant content remains a common practice in many natural product laboratories where their ease, speed and reproducibility are valuable traits in the screening of numerous samples. Therefore, the assays used must be selected specifically to best represent the overall antioxidant activity of a plant, encompassing different antioxidant mechanisms, compound polarity, rate of reaction, and so forth. There is a long and ever-growing list of *in vitro* antioxidant assays; so much so that covering all in the context of a single review would be nigh-impossible. However, despite the differences in names, reagents and methodology, *in vitro* antioxidant assays often fall under one of two categories:

2.1.1. Single electron transfer (SET)-based assays

SET-based assays involve a single redox reaction, with the oxidant being the indicator for endpoint measurement (Huang et al., 2005). Many common antioxidant assays are based on this mechanism, including TPC (total phenolic content), DPPH free radical scavenging activity, FRAP (ferric reducing antioxidant power), copper reduction assay (CUPRAC), and TEAC (Trolox equivalent antioxidant capacity) (Tabart, Kevers, Pincemail, Defraigne, & Dommes, 2009). Rather than actually measuring the "antioxidant" activity of a sample, these assays merely measure its reducing capacity (Benzie & Strain, 1999). These methods also typically lack a chain propagation step, which is crucial in lipid autoxidation. Hence, the results of these methods may not be relevant to radical chain-breaking antioxidant capacity (Antunes et al., 1999).

2.1.2. Hydrogen atom transfer (HAT)-based assays

HAT-based assays measure radical scavenging ability, and involve the use of a synthetic source of free radicals, an antioxidant, and an oxidizable molecular probe. These measure competitive reaction kinetics, and quantify antioxidant activity based on kinetic curves (Huang et al., 2005). Examples of HAT-based assays include TRAP (total radical-trapping antioxidant parameter), ORAC (oxygen radical absorbance capacity) and low-density lipoprotein (LDL) oxidation (Tabart et al., 2009).

Although both SET and HAT assays differ in terms of their reaction kinetics, the actual result (a stabilized free radical) is the same (Power et al., 2013). A detailed discussion of the mechanisms behind SET and HAT are outside the scope of this review, but there are two crucial differences between them:

1) Parameter measured: An antioxidant acting as a reducing agent, can do so by either donating a single electron, or by donating hydrogen atoms. SET-based assays measure the former, while HAT-based assays measure the latter (Huang et al., 2005). Although both SET and HAT mechanisms typically occur in parallel, the dominating mechanism would be affected by the physical and chemical properties of the antioxidant, the solvent system used and the pH at which the assay is conducted, which may potentially result in different kinetics and side reactions. Other factors that affect the dominating mechanism are the antioxidant's structure, bond dissociation energy and ionization potential (Power et al., 2013). Therefore, more than one property must be taken into account (Číž et al., 2010), particularly when analysing polyphenols given their different activities under different conditions (Prior, Wu, & Schaich, 2005).
2) Quantification of results: SET-based assays are quantified by measuring the change in the indicator's (oxidant) UV–Vis absorbance. While described as an "endpoint measurement" (Huang et al., 2005), SET reactions take a long time to reach completion and are thus calculated based on percentage decrease in a product within a specified period of time (Prior et al., 2005). Thus, the absorption endpoint may not represent a completed reaction (Proestos, Zoumpoulakis, & Sinanoglou, 2013). HAT-based assays on the other hand are quantified based on the kinetic curve between the probe, antioxidant and peroxyl radicals (Huang et al., 2005).

2.2. Determination of phenolic content

The most common antioxidants found in nature are phenols. This large group of over 8000 different compounds can be divided into two main categories depending on the number of phenol groups. The first category is simple phenols with one phenol group (a hydroxyl group attached to a phenyl ring), while the other is the polyphenols that have more than one phenol group. Polyphenolic compounds are of great research interest due to their reported antibacterial, antiviral, anti-allergic, anti-inflammatory, anticancer, and immunostimulant activities (Bansal et al., 2013). The "total

phenolic content" (TPC) assay using the Folin–Ciocalteu (FC) reagent is one of the most commonly used methods to quantify the phenolic content of a plant extract. Upon reaction with reducing agents, a blue-coloured complex is formed between the molybdenum and tungsten present in FC, which can then be measured through spectrophotometry (Ikawa, Schaper, Dollard, & Sasner, 2003). The high sensitivity, reproducibility and convenience of this SET-based assay has made it popular in routine screening of natural products (Huang et al., 2005).

However, this method has a number of drawbacks. FC was originally developed for protein analysis (Huang et al., 2005), and is consequently not specific to phenolic compounds, as it can react with a huge variety of other compounds including tryptophan, hydrazine, hydroxylamine, tertiary amine-containing biological buffers, N-hydroxyl compounds, N-amino compounds and N-heterocycles (Ikawa et al., 2003). In addition, although commonly known as the "total phenolic content assay", FC does not actually measure the phenolic content, but rather, it measures the sample's reducing capacity (Huang et al., 2005) and, at best, provides an estimation of a sample's phenolic content (Ignat, Volf, & Popa, 2011). In addition, its reactivity is also affected by the presence of other organic and inorganic reducing agents such as ascorbic acid and sulphites. FC has also been found to be very reactive towards –OH-containing amines (both aliphatic and aromatic) (Ikawa et al., 2003).

The widespread popularity of the FC reagent is in part due to its ease and rapidity, permitting it to be used for the screening of numerous samples in a relatively short period of time and at little cost. Compared to more laborious and costly methods such as the use of ion-exchange resins (Ikawa et al., 2003) or HPLC co-chromatography with known standards, it is easy to understand why this assay is so well-established despite its obvious flaws. Oddly enough, a standardized methodology for polyphenol determination with FC had been reported nearly half a century ago (Singleton & Rossi, 1965) but has fallen by the wayside in modern literature, despite giving more predictable and comparable results than the methodology commonly used today (Prior et al., 2005). Consequently, it can be argued that the TPC assay has limited value outside of initial screening (Clarke, Ting, Wiart, & Fry, 2013).

An alternative method, replacing the FC-based TPC assay, needs to be similarly uncomplicated, fast and affordable, yet with greater specificity for phenolics. One such method is based on the peroxidase-catalysed oxidation of phenols to phenoxyl radicals via hydrogen peroxide, which can then react with aromatic substrates to form coloured quinone-imine adducts, detectable via spectrophotometry. This method is similarly fast and affordable, while being specific to phenolic compounds and unaffected by compounds such as ascorbic acid or sulphites which would otherwise influence the results of the TPC assay (Stevanato, Fabris, & Momo, 2004).

Routine screening assays (be it TPC or peroxidase-catalysed oxidation) can also be combined with one or more different quantitative assays to give a more accurate estimate of the phenolic content. Flavanol content can be determined colourimetrically via the use of *p*-dimethylaminocinnamaldehyde (DMACA) (Feucht, Treutter, & Christ, 1996); flavones and flavonols, spectrophotometrically with $AlCl_3$ (Chang, Yang, Wen, & Chern, 2002; Wang, Gao, Zhou, Cai, & Yao, 2008). Anthocyanin content can be determined spectrophotometrically by pH-differentiation (Wrolstad & Giusti, 2001). Phenolic content can also be determined via HPLC co-chromatography with known standards. Although this method is more laborious, costly and specific only to the phenolic standards available at-hand, it remains the primary means of determining flavanone content (Escudero-López et al., 2013). It should be noted that although phenols remain the largest class of antioxidants found in nature, the overall antioxidant capacity of any sample should still consider the combined activities of other non-phenolic compounds (Tabart et al., 2009).

2.3. Free radical scavenging (FRS) activity

2.3.1. SET-based FRS assays, and the DPPH conundrum

Stable and commercially-available artificial radicals such as 1,1′-diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-azinobis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) form the basis of SET-based FRS assays. When these radicals are reduced by an antioxidant, the resulting change in colour can be monitored via spectrophotometry. Much like the TPC assay, these assays are popular in part due to their simple and rapid methodology, making them well-suited for screening. However, in addition to the general criticism that these *in vitro* methods do not sufficiently mirror *in vivo* models, these assays also use non-physiological radicals that bear little similarity to their biological counterparts, and are far more stable (Mishra, Ojha, & Chaudhury, 2012; Prior et al., 2005). These criticisms are often justified by emphasizing that these methods merely serve to rank the antioxidant activity of the samples *in vitro*, and not to reflect their actual antioxidant activity post-consumption (Villaño, Fernández-Pachón, Moyá, Troncoso, & García-Parrilla, 2007).

One of the most commonly used assays in evaluating the antioxidant activity of food and plant extracts is the DPPH free radical scavenging method. This otherwise-stable free radical is reduced from violet to yellow in the presence of antioxidants, and the change can be monitored spectrophotometrically (Milardović, Ivekovic, & Grabarić, 2006). The widespread application of this assay can be attributed to several factors: ease of performance, rapidness, automatability, reproducibility and usability at ambient temperature (thus preventing thermal degradation) (Kedare & Singh, 2011; Villaño et al., 2007).

Despite its widespread use, the DPPH assay itself suffers from numerous weaknesses. Varied DPPH concentrations, incubation times, sample volume, solvent systems and pH clearly show a lack of standardization, resulting in large differences in IC50 values even for standard antioxidants (Mishra et al., 2012; Sharma & Bhat, 2009). In addition, there has been increasing evidence that the relationship between the antioxidant concentration and DPPH radical scavenging activity is non-linear (Chen, Bertin, & Froldi, 2013). This has made it impossible to compare between laboratories that use different protocols.

Therefore, these factors need to be considered (and ideally standardized) when dealing with this assay. A DPPH concentration of 50 μM is recommended for good spectrophotometric accuracy, ideally in dim or dark conditions. The solvent recommended is methanol for less polar samples, or buffered methanol for more polar samples (Sharma & Bhat, 2009). The same solvent system should be used when attempting to compare different samples, as protic solvents such as methanol, ethanol and water result in an artificially higher FRS activity result when compared to non-protic solvents such as acetonitrile or ethyl acetate. This is due to the increased donation of protons to the DPPH radical following the regeneration of the catechol structure in phenols via nucleophilic attack on the corresponding *o*-quinones (Villaño et al., 2007).

Ascorbic acid is the recommended standard, as it achieves a steady state with DPPH within minutes, well within the typical half-hour reaction time of the assay (Mishra et al., 2012). Although a 30 min reaction time is the most common, other reaction times (like 5, 15, 20 and 60 min) have been previously reported (Boakye-gyasi, Ainooson, & Abotsi, 2011; Clarke et al., 2013; John & Shahidi, 2010; Murthy, Nataraj, & Setty, 2009). The kinetic-driven nature of the DPPH reaction with an antioxidant means that the rate of reaction differs depending on the nature of the antioxidant tested. For example, ferulic acid and BHT, two common standards for this assay besides ascorbic acid, can take two hours to fully react, while some compounds like curcumin can take up to six hours to fully react. Antioxidants with slow reaction kinetics

(exceeding an hour) tend to show significantly lower IC50 values at a fixed reaction time. This can lead to an under-representation of the antioxidant activity of a slow-reacting molecule (Mishra et al., 2012). The solution to this problem would be to monitor the reaction until its completion at set time intervals (Alañón, Castro-Vázquez, Díaz-Maroto, Gordon, & Pérez-Coello, 2011), or to report the results in terms of percentage reduction of DPPH (Kedare & Singh, 2011).

Often, FRS activity is reported in terms of IC50 or EC50, functionally similar terms in this context that both represent the amount of antioxidant required to scavenge 50% of the radicals present. This expression however, has a downside; it measures the effectiveness of an antioxidant at scavenging free radicals, but not how rapidly it scavenges them. This scavenging rate is crucial in biological systems, as naturally-occurring free radicals typically have short half-lives. Unfortunately, many "potent" antioxidant compounds with low IC50 values like flavonols (e.g. quercetin) and flavan-3-ols (e.g. catechin) are also fairly slow to react, and therefore not likely to effectively perform their roles as radical scavengers in a biological system (Villaño et al., 2007).

There are a number of proposed methods to improve the accuracy of the reported antioxidant activity based on the DPPH assay. For example, the antioxidant efficacy (AE) system proposed by Villaño et al. (2007), which takes into account the IC50 and scavenging rate of a compound, or the "antioxidant activity unit" (AAU) by Deng, Cheng, and Yang (2011) which enables comparison between samples treated with different concentrations of DPPH by calculating the number of moles required to scavenge one mole of DPPH radicals. Alternatively, the relative antioxidative capacity index (RACI) proposed by Sun and Tanumihardjo (2007) or antioxidative potency composition index (AOPI) by Seeram et al. (2008) can be used, where the both indices are a statistical integration of data from various *in vitro* assays, which are then expressed as a standardized mean score. By not relying on merely one antioxidant mechanism, these results give a more balanced report of the sample's antioxidant activity (Power et al., 2013).

In addition, DPPH quenching is not necessarily quantitative, as it can also be an equilibrium reaction (Stevanato et al., 2004) and is therefore reversible (Mishra et al., 2012), which is a problem given that DPPH is both the radical probe and the oxidant. DPPH can also be reduced by other reducing agents or other non-SET reactions. Reactivity would also be affected by steric accessibility, causing smaller molecules to seemingly exhibit superior antioxidant activity by virtue of their better accessibility, and vice versa (Prior et al., 2005). DPPH is also not able to detect all phenolic compounds, such as vanillin, p-hydroxybenzaldehyde, siringaldehyde, vanillic acid, p-coumaric acid, tyrosol, piceid and pterostilbene (Villaño et al., 2007) as these compounds may react with different kinetics, or simply not react with the DPPH radical at all (Mishra et al., 2012). The colours in highly-pigmented foods like red cabbage and cherries would also interfere with the absorbance of DPPH (Floegel, Kim, Chung, Koo, & Chun, 2011). Amperometry has been suggested as an alternative to overcome this drawback (Milardović et al., 2006).

In a similar vein with phenolic content determination, the accuracy of FRS determination can be improved by combining the data from two or more assays. Alternatives include the Trolox equivalent antioxidant capacity (TEAC) assay (Zulueta, Esteve, & Frígola, 2009); lipid peroxidation inhibition either through the β-carotene bleaching assay (Kumazawa et al., 2002) or thiobarbituric acid reactive substances (TBARS) assay (Laporta, Pérez-Fons, Mallavia, Caturla, & Micol, 2007); and the HAT-based oxygen radical absorbance capacity (ORAC) assay (Prior et al., 2005) which will be discussed in Section 2.3.2.

The TEAC assay is the most popular SET assay (Zulueta et al., 2009), likely due to the numerous drawbacks of the DPPH assay. This assay involves the scavenging of the aforementioned ABTS radical, and is often used in food samples (Floegel et al., 2011). Similar to the DPPH assay, this technique is fast and reproducible, but viable across a wide range of pH values (Lemanska et al., 2001), soluble in aqueous and organic solvents, and unaffected by ionic strength. This enables the determination of hydrophilic and lipophilic antioxidant capacity (Awika, Rooney, Wu, Prior, & Cisneros-Zevallos, 2003). Although this assay does not suffer from many of the DPPH assay's drawbacks, being a SET-based assay it still does not take into account reaction rates (Huang et al., 2005), making it nearly impossible to accurately quantify antioxidant capacity based on TEAC alone (Van den Berg, Haenen, Van den Berg, & Bast, 1999). TEAC results are also therefore strongly positively correlated with the DPPH assay, although the values obtained from the TEAC assay tend to be significantly higher (Floegel et al., 2011). In a large test of fifty of the most popular antioxidant-rich foods in the US diet, the TEAC assay has a better correlation with the ORAC assay when compared to the correlation between the DPPH assay and ORAC assay, suggesting that TEAC better reflects the antioxidant activity of food compared to the DPPH assay (Floegel et al., 2011). Regardless, both the DPPH and TEAC assays remain the most popular due to their convenient application (Floegel et al., 2011).

It is very common to see multiple SET-based assays being used simultaneously due to their ease, speed and reproducibility. However, doing so is relatively redundant. For example, DPPH or TEAC is often reported together with the TPC assay. These assays operate primarily on the same SET-based mechanism, which explains the excellent correlation observed between the results of both assays (Chandra et al., 2014). The same is true for the ferric reducing antioxidant power (FRAP); the ferric salt used (ferric tripyridyltriazine) has a comparable redox potential with ABTS. This means that similar compounds would react similarly in both the FRAP and TEAC assay (Prior et al., 2005). The FRAP assay is also redundant with the DPPH assay, given their reliance on similar antioxidant mechanisms (Clarke et al., 2013). Thus, any antioxidant activity reports based purely on SET-based assays, while not incorrect, merely represent a single facet of the sample's antioxidant activity; and should not be extrapolated to represent its overall antioxidant activity.

2.3.2. HAT-based FRS determination

As aforementioned, the antioxidant activity of a sample must be measured in more than one mechanism (Číž et al., 2010). The comparatively lower adoption of HAT-based assays compared to SET-based assays, such as DPPH and TEAC, can be attributed to the limitations of earlier HAT assays: the inhibited oxygen uptake (IOU) assay had low sensitivity and required an unfeasibly high oxygen pressure (Pryor, Strickland, & Church, 1988), and lipid oxidation inhibition assays often involved the formation of micelles, making it difficult if not downright impossible to accurately measure reaction processes (Huang et al., 2005). However, HAT-based assays are better analogues of *in vivo* action given that they measure antioxidant activity against peroxyl radicals, a biologically-significant radical involved in lipid peroxidation and autoxidation (Prior et al., 2005).

To overcome these issues, more recent HAT-based assays like ORAC, TRAP and crocin-bleaching assays, use molecular probes which enable easier reaction monitoring via UV or fluorescence. These molecular probes compete with antioxidants to bind to peroxyl radicals (generated via thermal decomposition of an azo compound). Although all three HAT-based assays operate on a similar general principle, the ORAC assay has been gaining popularity in biological (Cao & Prior, 1998), botanical (Prior & Cao, 2000) and nutraceutical samples (Balboa, Conde, Moure, Falqué, & Domínguez, 2013). This assay has undergone some modifications since its first introduction in 1993 (Cao, Alessio, & Cutler, 1993),

notably replacing the original fluorescent protein, B-phycoerythrin, with fluorescein, a synthetic non-protein probe to overcome the lack of specificity and batch inconsistencies of the original protein (Huang et al., 2005).

The ORAC assay is currently the preferred choice in the food and pharmaceutical industries (Power et al., 2013) as it can analyse samples with multiple ingredients and complex reaction kinetics, while still being able to analyse extracts, plasma and even pure phytochemicals (Cao et al., 1993; Huang et al., 2005). This assay can also directly measure hydrophilic and hydrophobic chain-breaking antioxidant capacities by H atom transfer (Cao et al., 1993), and can be easily adapted for high-throughput analysis with automated 48- or 96-well plate fluorescence readers (Huang, Ou, Hampsch-Woodill, Flanagan, & Prior, 2002). A major strength of this assay is that unlike many other assays which rely on the use of non-biological stable radicals, the ORAC assay uses peroxyl radicals, a biological species formed by the decomposition of 2,2′-azobis-2-methyl-propanimidamide dihydrochloride (AAPH), which would then react with a fluorescent probe, forming a non-fluorescent product (Power et al., 2013). Although the basic ORAC assay is limited to hydrophilic substrates due to the low solubility of the fluorescent probes in hydrophobic environments, this assay can be easily adapted to measure the antioxidant activity of lipophilic compounds by substituting fluorescein with 4,4-difluoro-3, 5-bis(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene (BO-DIPY 665/676) and 2,2 the low solubdimethylvaleronitrile) (AMVN) as the peroxyl radical generator (Ou et al., 2002).

In comparison with SET-based assays like DPPH or TEAC, the HAT-based ORAC assay gives considerably different results even when analysing the exact same compounds, further emphasizing the need for both SET and HAT-based assays to give a better overall estimate of a sample's antioxidant activity. While the DPPH and TEAC assays are well-correlated with one another (due to their similar mechanisms), neither assay correlates well with the ORAC assay which often reports a higher antioxidant activity for phenolic compounds. However, ORAC reports lower antioxidant activities for some non-phenolic antioxidants such as ascorbic acid and reduced glutathione. ORAC is consistently more sensitive than its SET-based counterparts (with DPPH showing the least sensitivity), and can detect the antioxidant activity of compounds such as naringenin and hesperidin which show almost no antioxidant activity with the DPPH or TEAC assay. This trait can be attributed to ORAC's combined measurement of both the inhibition time and degree of inhibition, coupled with the completion of the reaction (Tabart et al., 2009). It should be noted that the increased sensitivity of the ORAC assay does not mean that it necessarily reports higher values compared to TEAC, as it is still dependant on the chemical composition of the samples. For example, the ORAC assay showed comparatively lower antioxidant activity in crude algal extracts when compared to the TEAC assay (Balboa et al., 2013). The ORAC assay is also weakly correlated with the FRAP assay (Floegel et al., 2011), further reinforcing the difference in results observed between SET and HAT-based assays.

The popularity of ORAC over TRAP and crocin-bleaching is likely due to the limitations of the latter two assays. TRAP relies on the lag phase of the kinetic curve for quantitation, while ORAC relies on the overall area under the kinetic curve. In practice, not all antioxidants have a lag phase; and measurements based on lag phase alone are prone to overestimation in weaker antioxidants (which have a longer lag phase) (Huang et al., 2005) Therefore this assay is prone to underestimation of overall antioxidant activity as it disregards all antioxidant activity beyond the lag phase (Prior et al., 2005). The TRAP assay is also less sensitive than ORAC (Číž et al., 2010). Crocin-bleaching on the other hand is easily contaminated by many compounds (notably carotenoids) that absorb at the monitored wavelength (450 nm). In addition, crocin is typically sold as an extract of saffron, and not as a pure compound. It is therefore subject to variability, thus limiting its value for quantification studies (Huang et al., 2005). The ORAC assay is not without its weaknesses, as it only measures antioxidant activity against peroxyl radicals ($RCOO^{\cdot}$) and disregards the five other major reactive oxygen species.

2.4. The six reactive oxygen species

There are six major reactive oxygen species: the peroxyl radical ($RCOO^{\cdot}$), superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), hydroxyl radical ($HO^{\cdot}$), and peroxynitrite ($ONOO^-$) (Huang et al., 2005). Given that antioxidants operate differently against different radical species (Prior et al., 2005), an overall antioxidant activity assessment should ideally cover the scavenging activity against these six radicals. The incredible breadth of this particular topic and numerous assays that can be used to measure each reactive oxygen species make it unfeasible to be covered in this review in great detail. Table 1 summarizes some of the various options available.

2.5. Antioxidant activity: making the right call

It is unfortunately unrealistic to expect a single assay to be able to determine the total antioxidant activity of a sample (Power et al., 2013), although this is often reported in literature (Huang et al., 2005). It must be noted that the activities measured by these assays are only reflective of their activity under those specific conditions. The incomparability of the data from these various antioxidant assays, combined with the increasing number of different methods to determine antioxidant assays make it exceedingly difficult for research groups and industries (particularly those in the food and nutraceutical industries) to compare and evaluate the data presented (Huang et al., 2005) and great caution must be taken in interpreting the antioxidant activities based on different assays (Floegel et al., 2011). This is further due to the multiple mechanisms by which a single antioxidant can act in a system, and their differing efficacy against different radical or oxidant sources (Prior et al., 2005).

The key here is diversity in the variable measured by the assays. Ideally, a project dealing with antioxidant activity assessment should have a mix of SET-based and HAT-based assays, encompassing several different radical types. Many of these techniques are spectrophotometric, which are incapable of quantifying or identifying individual phenolic compounds, a role often performed via RP-HPLC, MS or NMR (Ignat et al., 2011). Isolation and identification of phytochemicals remains an important aspect in natural product research, as understanding the composition and properties of the individual compounds gives great insight into the bioactivity of a particular sample (Abu-Reidah, Arráez-Román, Segura-Carretero, & Fernández-Gutiérrez, 2013).

Although it could be tempting to discredit spectrophotometric techniques in favour of more laborious and specific methodology, spectrophotometric techniques still have their place as the isolation of individual compounds is not only slow, costly and inefficient, but also disregards any interactions (synergistic or antagonistic) between the various compounds present in a sample (Tabart et al., 2009). *In vitro* assays remain useful research tools, and are still able to help assess the overall antioxidant activity of a sample when used in combination with bioavailability and biomarker assays (Power et al., 2013).

Table 1
Assays used to evaluate the antioxidant activity against the six reactive oxygen species.

Species	Assays	Notes and citation
Peroxyl radical (RCOO•)	ORAC TRAP Crocin bleaching	HAT-based, discussed in Section 2.3.2
	β-carotene bleaching	Inhibition of β-carotene bleaching by scavenging of RCOO• monitored spectrophotometrically at 470 nm. Antioxidant activity affected by pH, metal content and ratio of antioxidant solution to β-carotene/linoleic acid (Dawidowicz & Olszowy, 2010)
Superoxide anion ($O_2^{\bullet-}$)	Gas chromatography	Balboa et al. (2013)
	Reduction of nitrobluetetrazolium (NBT) or cytochrome c	Spectrophotometric analysis at 530–560 nm, observing the reduction of NBT to formazan by $O_2^{\bullet-}$. A decrease in absorbance (presence of formazan) indicates increased superoxide scavenging activity (Jena, Ratha, & Kar, 2013). Equilibrium cannot be achieved while $O_2^{\bullet-}$ is generated continuously in the assay, thus requiring careful interpretation of the data (Frankel & Meyer, 2000)
Hydrogen peroxide (H_2O_2)	Fluorescence	Oxidation of scopoletin with horseradish peroxidase into a non-fluorescent product (Balboa et al., 2013)
	Titanium (IV)	Precipitation of the Ti-H_2O_2 complex can be dissolved in sulphuric acid and measured at 410 nm (Wang & Jiao, 2000)
Singlet oxygen ($^1O_2$)	Photooxidation	A carotenoid is mixed with a photosensitizer (e.g. Rose Bengal), and photooxidation by singlet oxygen is monitored via UV–Vis HPLC at 445 nm (Ramel et al., 2012)
Hydroxyl radical (HO•)	Fluorescence	Degradation of 2-deoxyribose caused by hydroxyl radicals (generated via an *in vitro* Fenton's reaction) can be spectrophotometrically monitored at 520 nm (Nagai, Myoda, & Nagashima, 2005)
	Ferrous ion chelating (FIC)	Measures Fe^{2+} chelating potential of the sample. Chelation of transition metals is the most important form of secondary antioxidant activity to prevent catalysed production of hydroxyl radicals (Balboa et al., 2013)
	Total oxidant scavenging capacity (TOSC)	HAT-based. Reliance on gas chromatography makes it not readily adaptable for high-throughput analysis, and antioxidant activity is not linearly correlated with antioxidant concentration, thus making it difficult to compare between different samples (Winston, Regoli, Dugas, Fong, & Blanchard, 1998)
Peroxynitrite ($ONOO^-$)	Total oxidant scavenging capacity (TOSC)	Also able to measure antioxidant activity against hydroxyl radicals (see above)

3. Antibacterial activity

The rise of antibiotic-resistant microorganisms has given extra impetus in the search for novel antibacterial compounds. Numerous alkaloids, flavonoids, glycosides, terpenes, tannins and polyphenols from plant origins have been shown to exhibit antibacterial activity. Many have also been known to exhibit synergistic effects with existing antimicrobial drugs (Ncube et al., 2008). In the literature it is common to see both the antioxidant and antibacterial activity reported concurrently, particularly when bioactivity-guided fractionation is involved. It is therefore similarly crucial that the methods used for antibacterial testing also be standardized and optimized to ensure the accuracy of reports, and the appropriate selection of the most desirable fractions.

3.1. Choice of methodology

The reported antibacterial activity of flavonoids is often widely conflicting. This is mainly due to the different methods used to assess the antibacterial activity of these compounds (Cushnie & Lamb, 2005). *In vitro* antibacterial testing is still crucial in the screening process, and several bodies such as the Clinical and Laboratory Standards Institute (CLSI) [formerly known as National Committee for Clinical Laboratory Science (NCCLS)], British Society for Antimicrobial Chemotherapy (BSAC) and the European Committee for Antimicrobial susceptibility testing (EUCAST) have approved guidelines for the testing of bacterial susceptibility on conventional drugs that can be slightly modified to better suit plant extracts.

One of the earliest and most widely-used antibacterial screening techniques is the disc diffusion technique; popular due to its ease of preparation, low cost and no need for specialized equipment (Osato, 2000). This method involves the loading of an antimicrobial testing disc with a compound or extract of choice which is then placed onto an agar medium inoculated with a lawn culture of bacteria. Following the diffusion of compounds from the disc into the surrounding agar, any antimicrobial property is visible by the presence of a "clear zone" where the growth of bacteria has been inhibited (Lee et al., 2010). The diameter of this zone is directly related to the polarity of the compounds, its concentration and molecular weight. Thus, although disc diffusion is ideal for highly polar compounds, slightly soluble compounds can still be tested, albeit resulting in smaller clear zones. However, non-polar compounds may fail to diffuse, resulting in false negatives (Ahmed et al., 2013).

Great care must be taken to not correlate the zone of diffusion with the strength of the antibacterial activity – a misconception that still exists even in recent scientific literature. This would be true if the same compound(s) were tested, but even then it would only show comparative susceptibility of different bacterial species to that compound – it would not be possible to compare the antibacterial activity of different samples, as the different compounds present will diffuse at different rates (Ncube et al., 2008). There is also a likelihood that potent antibacterial polyphenols may have a low rate of diffusion (Cushnie & Lamb, 2005). Ultimately, the disc diffusion method is a qualitative test, and its results should not be used for quantitative purposes as it is impossible to determine the MIC, minimum bacteriocidal or minimum bacteriostatic concentrations using this method (Ncube et al., 2008). It does, however, remain a useful method for quick and simple qualitative screening (King, Dykes, & Kristianti, 2008).

A suitable method for the quantification of antibacterial activity that has been gaining popularity is the broth microdilution method, which involves serial dilution of the tested sample in broth (typically Mueller–Hinton broth), which is then inoculated with bacteria in a 96-well microtitre plate (King et al., 2008). Unlike the disc diffusion assay, this assay can be used to determine the MIC of a sample and remains one of the few techniques able to determine the minimum bactericidal concentration (Osato, 2000). Results between the disc diffusion and broth microdilution method are not always comparable (King et al., 2008), but the results obtained from the latter method are considered more accurate (Othman et al., 2011).

Broth microdilution, however, is not without its drawbacks. Heat-labile water-based extracts and hydrophilic compounds can only be reliably sterilized via membrane filtration, which may affect the activity if the compounds are adsorbed onto the membrane (EUCAST, 2003). This method is also unsuitable for testing highly non-polar compounds and extracts. Although more toxic solvents such as methanol and acetone can be used as solvents for water-insoluble compounds (up to 2% final concentration without being toxic to bacteria) (Mathekga, Meyer, Horn, & Drewes, 2000), DMSO is a popular alternative given its comparatively lower toxicity. However, it is still important to determine the DMSO tolerance of the tested bacteria before proceeding with further MIC determination. Some bacteria may be inhibited by concentrations barely exceeding 2% DMSO (Miyasaki et al., 2013), although some may not be noticeably affected even at over 30% DMSO (Shah, Modi, Shukla, & Lahiri, 2014).

Regardless of the solvent used, some non-polar compounds would tend to precipitate given the aqueous nature of this method. Precipitation of less-polar polyphenols like flavonoids would lead to diminished contact between the compounds tested and the bacteria, thus limiting their activity (Cushnie & Lamb, 2005). This issue can be reduced by the addition of Tween 80 to enhance the solubility of compounds. The concentration Tween 80 used tends to be low, ranging from less-specific concentrations of "a few drops" (Khan et al., 2007) to more specific concentrations such as 0.02% (Reis et al., 2012), 0.1% (Ji, Luo, & Yan, 2008) and up to just below 5% (Ahmed et al., 2013). Oils on the other hand may require up to 10% Tween 80 (Matasyoh, Maiyo, Ngure, & Chepkorir, 2009). Determination of the MIC can also be difficult due to the interference of turbid or highly coloured compounds, making visual or spectrophotometrical measurements inaccurate, if not downright impossible. Colourimetric indicator methods are an alternative solution to this issue, and also help minimize the ambiguity of visual observation (Ncube et al., 2008).

The agar dilution assay is an alternative method that circumvents most of the issues with the broth microdilution method. It involves mixing the sterilized sample with the agar (usually Mueller–Hinton agar) and inoculating it with bacteria. This method is also quantitative (Silva, Simas, Batista, Cardarelli, & Tomassini, 2005), but considerably more tedious than broth microdilution, particularly when dealing with large sample numbers. Therefore, agar dilution is not necessarily an improvement upon the broth microdilution method, but merely an alternative option. In recent years, the use of multi-channel oxygen metres with disposable oxygen electrode sensors have also been used to assess the antimicrobial activities of hydrophobic compounds on aerobic bacteria. This is done by directly measuring their oxygen consumption, thus bypassing all needs for spectrometric observation while simultaneously being less labourious than the agar dilution assay (Kitahara et al., 2006). Relating oxygen consumption to cell viability may however be potentially inaccurate, as bacterial aggregation would result in a disproportionate reduction oxygen consumption (Cushnie & Lamb, 2011).

3.2. Standardization in antibacterial activity assessment

Regardless of the method chosen, the inoculum size is the most crucial variable in susceptibility testing (NCCLS, 2000a) as an inappropriately light inocula would give falsely lower MIC values, and vice versa (Wiegand, Hilpert, & Hancock, 2008). While the 0.5 McFarland turbidity standard (1.5×10^8 CFU/ml) (Abachi, Khademi, Fatemi, & Malekzadeh, 2013; Barku, Opoku-boahen, Owusu-ansah, Dayie, & Mensah, 2013) is often used as a 'visual yardstick' to save time when handling a large number of samples (Wiegand et al., 2008), it is still advisable that inoculum sizes be more accurately standardized. Common concentrations for antibacterial testing are usually approximately 10^6 CFU/plate for disc diffusion (Proestos et al., 2013), 5×10^5 CFU/ml for broth macrodilution (Ahmed et al., 2013; Novy, Rondevaldova, Kourimska, & Kokoska, 2013; Wiegand et al., 2008), 10^4 CFU/spot for agar dilution (Wiegand et al., 2008), while 7×10^5 CFU/ml is preferred for the agar diffusion method (Jagtap & Bapat, 2013).

Despite the imperative importance of the inoculum size, the CFU used in literature does not appear to be consistent; for example, 10^6 CFU/ml (Katalinic et al., 2013) and 10^8 CFU/ml have been used for broth microdilution (Hasan et al., 2013; Martins et al., 2013). These discrepancies continue to occur despite the initial release of the CLSI guidelines for agar dilution, broth microdilution and broth macrodilution well over a decade ago (NCCLS, 2000b). Worse still, even in recent literature, the inoculum size is sometimes overlooked and not reported. Regardless of the inoculum size used, the bacterial suspension should be used within 30 min after it has been adjusted, to avoid significant changes in cell number (Wiegand et al., 2008).

Another crucial factor is the method by which the MIC and MBC values are determined. The MIC value is of particular importance, being generally used as the standard for expressing the susceptibility of an organism to an antimicrobial agent. Unfortunately, the determination of the MIC is inconsistent, with variations between different researchers and standardization bodies. According to the BSAC, the MIC can be defined as the lowest concentration capable of inhibiting visible growth of a microorganism after overnight incubation (Andrews, 2001); while CLSI (2006) and EUCAS (2000) define the MIC as the lowest concentration (mg/l) able to prevent growth under a defined period of time under defined conditions. The exact MIC breakpoints that define if a microorganism is susceptible, treatable or nigh-untreatable vary according to the particular species; taking into account clinical data and MIC distributions of relevant species, as well as the pharmacodynamics and pharmacokinetics of the antimicrobial agent used (Wiegand et al., 2008). Given the role of MIC as a means of resistance surveillance, even slight changes in the MIC of a microorganism is clinically relevant (Wiegand et al., 2008) – it is therefore imperative that the MIC values reported are based on the same standards.

MBC determinations are less common compared to MIC determination (Andrews, 2001), but remain of interest since killing infectious bacteria is often preferred over merely inhibiting their growth (Cushnie & Lamb, 2011). There is some controversy regarding whether phenolic compounds are bacteriocidal or bacteriostatic following the discovery that antibiotic-sensitive and antibiotic-resistant strains of *Staphylococcus aureus* could form pseudomolar aggregates, thus falsely reducing the observed CFU in viable counts (Stapleton et al., 2004). Various phenolic compounds have been reported to cause aggregation, including galangin (Cushnie, Hamilton, Chapman, Taylor, & Lamb, 2007) and epicatechin gallate (Stapleton, Shah, Ehlert, Hara, & Taylor, 2007). This would affect the interpretation of MBC and time-kill studies (Cushnie & Lamb, 2005) as it can no longer be assumed that reductions in CFU are a result of decreased viability (Cushnie & Lamb, 2011).

4. Conclusion

As aforementioned, the antioxidant and antibacterial activity of a particular sample are often concurrently reported, particularly when bioactivity guided fractionation is involved. Therefore, scientists need to be wise in their selection of methodology for assessing *in vitro* antioxidant and antibacterial activities, not only to ensure the integrity of their reported results, but also to ensure their further isolation, identification and characterization work is well-targetted.

The most accurate estimate of a plant sample's antioxidant activity *in vitro* would be a combination of assays, taking into account two main factors: the antioxidant mechanism, and the targeted reactive species. Therefore, it is highly recommended that studies focusing on the *in vitro* antioxidant activity of a plant sample account for both the SET and HAT mechanisms, in addition to measuring the scavenging activity against several different reactive species. Although some of the long-standing assays like the TPC and DPPH assay suffer from a number of drawbacks, the data gleaned from such assays are still useful as long as they are interpreted and represented correctly.

In the evaluation of *in vitro* antibacterial activity, a key deciding factor regarding the methodology chosen would be the solubility of the compounds tested. Broth microdilution works best with hydrophilic compounds, however less polar extracts can still be solubilized by the addition of small amounts of Tween 80 or DMSO. Alternatively, the agar dilution assay could be used for these less polar extracts. Overall, both the broth microdilution and agar dilution assays give the most reliable results and are capable of determining the MIC and MBC of a particular sample. Regardless of the method chosen, it is nevertheless crucial to standardize inoculum sizes, and ensure that the MIC and MBC is clearly defined.

The sheer breadth and depth of all the *in vitro* antioxidant and antibacterial assays for testing plant samples remains too broad to be covered within the scope of a single review. However, this review has hopefully given an insight into some of the key points to consider when choosing such assays.

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