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Research Article

Relation between Silver Nanoparticle Formation Rate and Antioxidant Capacity of Aqueous Plant Leaf Extracts

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Correlation between the antioxidant capacity and silver nanoparticle formation rates of pomegranate (*Punica granatum*), quince (*Cydonia oblonga*), chestnut (*Castanea sativa*), fig (*Ficus carica*), walnut (*Juglans cinerea*), black mulberry (*Morus nigra*), and white mulberry (*Morus alba*) leaf extracts is investigated at a fixed illumination. Silver nanoparticles formed in all plant leaf extracts possess round shapes with average particle size of 15 to 25 nm, whereas corresponding surface plasmon resonance peak wavelengths vary between 422 nm and 451 nm. Cupric reducing antioxidant capacity technique is used as a reference method to determine total antioxidant capacity of the plant leaf extracts. Integrated absorbance over the plasmon resonance peaks exhibits better linear relation with antioxidant capacities of various plant leaf extracts compared to peak absorbance values, with correlation coefficient values of 0.9333 and 0.7221, respectively.

1. Introduction

Among the broad range of nanoparticles (NPs) which exhibit unique properties in relation to their sizes, silver nanoparticles (AgNPs) have found widespread medical applications since they exhibit antibacterial [1–8], antiviral [9], and antifungal [10] effects. Synthesis of AgNPs is a multistage process [11] involving first the reduction of Ag^+ to Ag^0 by capturing an electron from the surrounding molecules which readily donate electron. During nucleation, reduced silver atoms aggregate to seed the growth process, which is then stabilized in the final stage to form stable AgNPs [12].

AgNPs can be mass produced using synthetic [13] or biological [1–11, 14–17] techniques. In the former, specific reagents such as sodium borohydride, sodium citrate, and ascorbic acid are employed in a strict protocol for the reduction of Ag^+ and the stabilization of AgNPs so that AgNPs with well-defined shapes and sizes are obtained. In biological techniques which incorporate the use of bacteria [14], fungi [15], yeast [16], algae [17], and plants [1–11], however, both reduction and stabilization processes succeed in a mixture of biomolecules whose content depends on initial biological materials. Use of plant leaves for the synthesis of AgNPs has been popular since it offers a facile and affordable alternative to synthetic techniques as it can yield well-characterized nanoparticles [7]. Despite its advantages, a major drawback of this technique is the lack of a clear understanding of the processes ongoing during the formation and the stabilization of AgNPs. Although it is still under dispute, various biomolecules existing in aqueous plant leaf extract such as steroids, polyphenols, polysaccharides, terpenoids, stearic acid, thiols, ascorbic acid, and flavonoids have been proposed to take role in AgNP formation [5, 6]. A majority of such biomolecules known as antioxidants [18, 19] are, in fact, successfully employed in the chemical synthesis of AgNPs.

Utilization of above-mentioned biomolecules in the chemical synthesis of AgNPs is not surprising since antioxidants have the capacity to alter the oxidation level of metal ions by supplying electrons. Most plants contain numerous molecules with different antioxidant capacities [18, 20, 21]. Although relative amounts of such biomolecules differ between the parts of plant and show seasonal variations [5, 6], leaves are known to include significantly high concentration of antioxidants [18, 19]. Different spectroscopic

methods based on hydrogen atom or electron-transfer reactions [22, 23] have been developed for determining total antioxidant capacity (TAC) of biological materials such as food, beverages, and plant extracts [24, 25]. A reliable, yet simple, reference method needs to be developed for TAC measurements of biological materials. TAC of aqueous plant leaf extracts simply defines the electron supplying capacity of the extracts and could be related to AgNP formation rate in that specific extract since AgNP formation relies on the reduction of Ag^+ in which the electron is supplied by the molecules in the extract.

Use of NPs for TAC measurements is delayed until the pioneering work of Scampicchio et al. [26] who employed gold nanoparticles. AgNP-based technique with improved sensitivity and reliability is employed and that referring to peak absorbance value of surface plasmon resonance (SPR) band later is proposed for TAC measurement of polyphenols [24]. An assay based on CeO_2 nanoparticle formation rate is also proposed for rapid detection of food antioxidants [27]. Although $Ag^+ + e^- \leftrightarrow Ag^0$ reaction is known to be light sensitive, illumination in above-mentioned works appears to be uncontrolled. On the other hand, it was recently shown that irradiation significantly influences AgNP formation rates in plant leaf extracts [6]. Therefore, relation between the antioxidant capacities of plant leaf extracts and AgNP formation rates under constant illumination needs to be addressed to keep its effects unvarying.

A frequently employed practice of time-tracking the absorbance value at a specific wavelength, for example, the wavelength at SPR peak, turns out to be feasible for many purposes [5, 6], although it might be misleading since it does not reflect the response of nanoparticles in the whole size range. In fact, as the absorbance around the SPR peak wavelength remains constant or decreases, absorbance at long wavelengths is enhanced due to developing shape anisotropy, aggregation, and/or increase in particle size especially at prolonged contact time [5]. Therefore, integrated absorbance covering the whole SPR band rather than the one at a specific wavelength could better represent the situation.

The aim of this work is to explore a correlation between TAC and the area under the SPR band of AgNPs synthesized in different plant leaf extracts at constant illumination to address whether SPR band could be exploited as a means of estimating TAC of complex plant extracts. Results indicate a more pronounced correlation between TAC and area under the SPR band in comparison to peak absorbance.

2. Experimental Details

Pomegranate (*Punica granatum*), quince (*Cydonia oblonga*), chestnut (*Castanea sativa*), fig (*Ficus carica*), walnut (*Juglans cinerea*), black mulberry (*Morus nigra*), and white mulberry (*Morus alba*) leaves are collected from the campus area of Uludag University, Bursa, Turkey, at an altitude of 117 m. Leaves are shadow-dried, grinded, and sieved prior to use. 1.0 g of sieved leaf sample is admixed with 250 mL distilled water and stirred at room temperature for an hour on a magnetic stirrer prior to filtration. Equal volumes of 2 mM $AgNO_3$ solution and filtered leaf extracts are mixed under the illumination of a 7 W (295 lumens) white fluorescent light source (Megaman 4U108i), which is chosen for its low thermal radiation, at room temperature. UV-visible (UV-Vis) spectra of the mixtures are recorded at 15 minute intervals for 120 minutes in total using a Hitachi U3900H double-beam spectrophotometer in the 200–800 nm range at a resolution of 0.5 nm.

Cupric reducing antioxidant capacity (CUPRAC) method, which is an electron-transfer-based technique originally developed to measure TAC of natural biological samples, for example, human blood plasma [25], is chosen to determine the TAC of the plant leaf extracts. CUPRAC is chosen as the reference method to measure the TAC of the plant leaf extracts since it has distinct advantages over other electron-transfer-based techniques [28]. It is simple, reproducible over a wide concentration range, suitable for around pH 7, as it is the case for most of the plant leaf extracts, rapid for most common flavonoids, where antioxidant compounds are selectively oxidated without affecting sugars and citric acid commonly contained in food stuff, whereas the employed reagents are stable [24, 25, 28]. The method relies on reduction of Cu^{+2} to Cu^+ in neocuproine (2,9-dimethyl-1,10-phenanthroline) complex by the antioxidants and measuring the absorbance peak of Cu(I) neocuproine at 450 nm [25].

To determine TAC of plant leaf extracts, equal volumes (1 mL) of 1.0×10^{-2} M Cu(II) chloride solution, 1.0 M ammonium acetate buffer (pH 7), and 7.5×10^{-3} M neocuproine solution are mixed together prior to adding 1.1 mL of the diluted sample (0.2 mL or 0.3 mL aqueous plant leaf extracts are brought to 1.1 mL by the inclusion of distilled water) to obtain a total volume of 4.1 mL. The mixture is then kept at room temperature in an air-tight vessel for 30 minutes prior to measuring its absorbance at 450 nm against the reference solution, which contains the same ingredients as the sample solution except that the extract is replaced with distilled water of equal volume. Averages of three measurements at two dilution ratios are taken.

TAC of different samples are presented as their trolox ($C_{14}H_{18}O_4$, FW: 250,26 g/mol) equivalents per dried leaf weight (i.e., trolox/dried leaf weight). Trolox is a reference material in transfer electron antioxidant capacity method which was first proposed by Miller et al. in 1993 [29]. It is commonly used for the antioxidant capacity measurements and structurally compatible with most of natural antioxidants. A calibration curve is obtained by following the above procedure for different volumes, for example, 25 μL, 50 μL, 100 μL, 200 μL, and 300 μL, of 1.0×10^{-3} M standard trolox solution (0.25026 g/L) as samples. Absorbance of each trolox solution measured at 450 nm is then plotted against the trolox weight in Figure 1 and the curve is used to determine the trolox equivalent weight of the TAC for each plant leaf extract.

A Malvern Zetasizer NanoZS particle size analyzer is employed to determine the size distribution of as prepared AgNPs. A proportion of biosynthesized AgNPs are centrifuged at 10000 rpm for 15 minutes. Precipitated AgNPs are then washed three times with deionized water in an ultrasonic bath to remove the organic constituents. Small amounts of such triple-washed AgNPs are transferred on

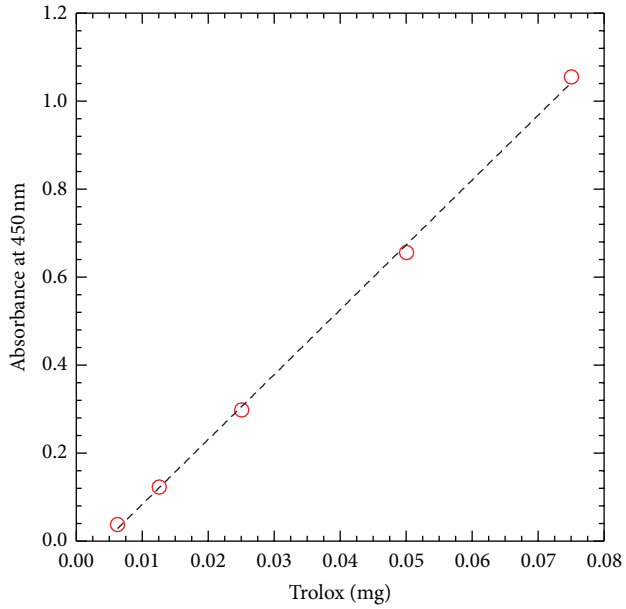


Figure 1: Variation of absorbance at 450 nm of trolox solution with its trolox content. Dashed line represents the best linear fit to data points.

a Formvar-coated copper grid for morphological investigations in a Zeiss Leo 906E transmission electron microscope (TEM).

3. Results and Discussions

TEM investigations of all the samples prepared indicate that AgNPs are round shaped and have average particle sizes of approximately 15 nm. TEM micrograph and particle size distribution of AgNPs synthesized using mulberry leaf extract are presented in Figures 2(a) and 2(b), respectively, as typical of all. Particle size distributions reveal a single peak at approximately 15 nm with an extended tail up to 30 nm suggesting that AgNPs are monodispersed despite the fact that several clusters are visible in Figure 2(a).

UV-Vis spectra of the mixture of plant leaf extracts and $AgNO_3$ solution reveal strong SPR bands around 430 nm, as being characteristic of spherical AgNPs, Figure 3(a) [30]. SPR peak maxima for the investigated plant leaves occur at 422.0 nm, 430.5 nm, 440.0 nm, 442.5 nm, 442.5 nm, 445.0 nm, and 451 nm for pomegranate, quince, chestnut, fig, walnut, black mulberry, and white mulberry leaf extracts, respectively, Figure 3(b). Although time variation of SPR peak absorbance in Figure 3(c) reveals that peaks intensify with time, their positions remain relatively fixed, Figure 3(b). SPR bands appearing around 600–700 nm at prolonged times for pomegranate and chestnut leaf extracts in Figure 3(a) can be due to large nanoparticles with spherical shape or nanoparticles with considerable shape anisotropy and/or aggregates [5, 30]. TEM investigations and particle size distribution analysis do not support the existence of either large spherical AgNPs or AgNPs with strong shape anisotropy (Figure 2); they rather

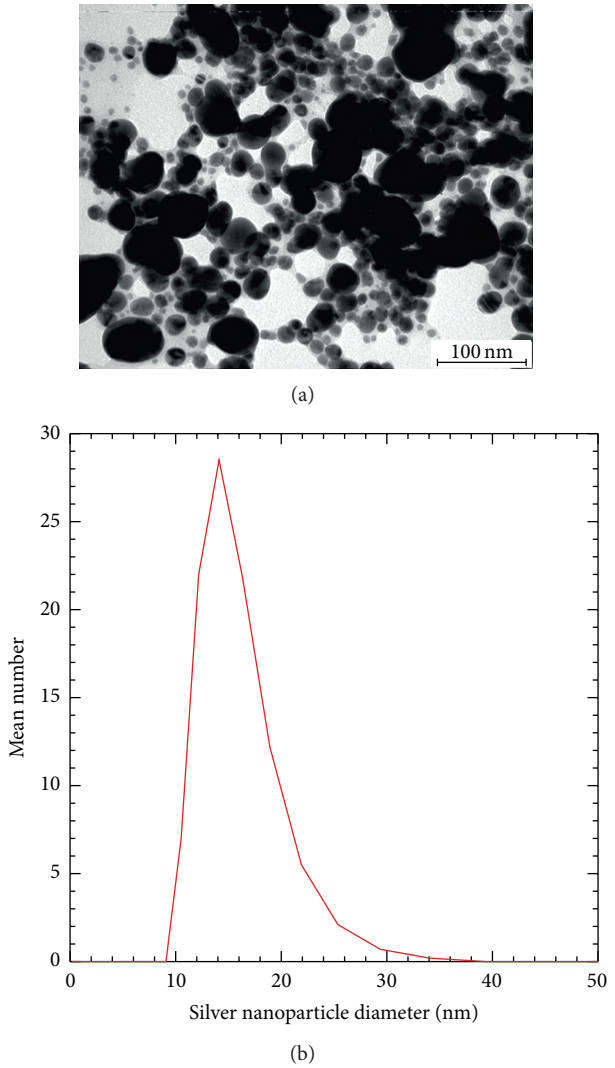


Figure 2: TEM micrograph (a) and the particle size distribution (b) of AgNPs synthesized using mulberry leaf extract.

suggest that SPR bands around 600 nm in Figure 3(a) may be due to aggregates of small spherical AgNPs.

Figure 3(c) displays the diversity in the nucleation and growth processes of AgNPs among different plant leaf extracts. Although peak absorbance reaches saturation in 120 minutes for fig and chestnut leaf extracts, it is not yet reached for pomegranate, walnut, quince, and mulberry leaf extracts in the same period, Figure 3(c). Among the plant leaves studied, AgNP formation processes in mulberry leaf extracts appear to be the slowest of all. They reveal almost linear variation with time, suggesting that nucleation will still proceed for some time after 120 minutes. Although AgNP nucleation in mulberry leaf extracts is in progress at 30 minutes, nucleation appears to be completed for the others as the growth of AgNPs in chestnut and fig leaf extracts is almost completed.

Peak wavelengths in Figure 3(b), which reflect the average AgNP sizes [6], vary between 423 nm and 454.5 nm with time. Pomegranate leaf extract which displays the strongest

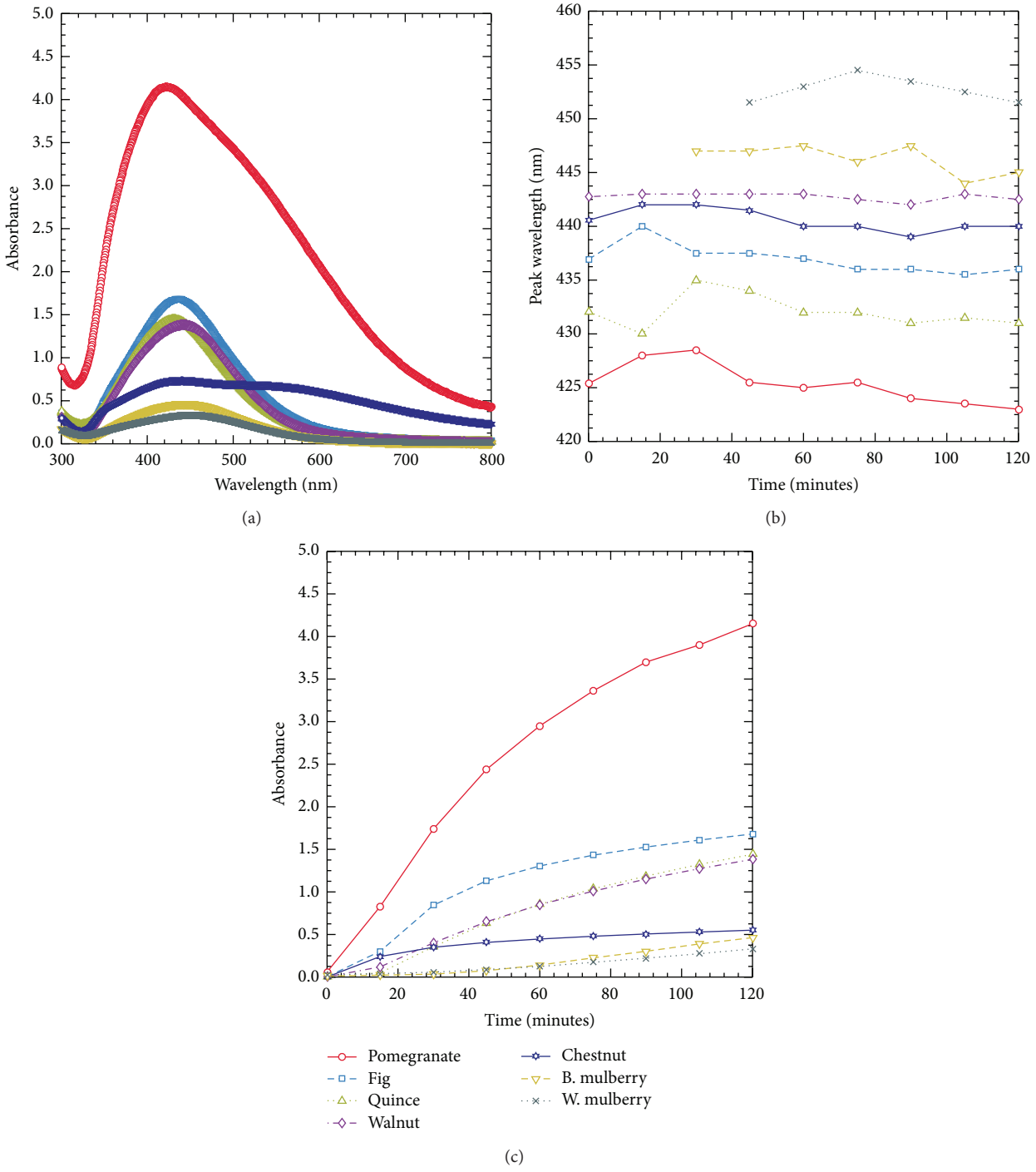


FIGURE 3: UV-Vis spectra of the mixtures of pomegranate, quince, chestnut, fig, walnut, mulberry leaf extracts, and 2 mM $AgNO_3$ solutions recorded 120 minutes after the mixing (a). Time variations of SPR peak wavelengths (b) and peak absorbance (c) for the mixtures in (a).

peak in Figure 3(a) and the highest rate of AgNP formation of all (Figure 3(c)) have the SPR peak at the smallest wavelength, 422 nm in Figure 3(b). SPR peak for mulberry leaf extracts however appears at highest wavelengths, 452 nm and 455 nm in Figure 3(b), while exhibiting the weakest peak (Figure 3(a)) and the slowest AgNPs formation rates of all (Figure 3(c)). Walnut leaf extract reveals the most time-stable SPR peak with a standard deviation of 0.38 while pomegranate, quince, fig, black mulberry, white mulberry, and chestnut leaf extracts have standard deviations of 2.00, 1.66, 1.45, 1.35, 1.17, and 1.12, respectively.

Peak and integrated absorbance values of the SPR band of the mixtures of plant leaf extracts and $AgNO_3$ solutions are plotted against the antioxidant capacity of the corresponding plant leaf extracts in Figures 4(a) and 4(b), respectively. Integrated absorbance over the SPR bands between 300 nm

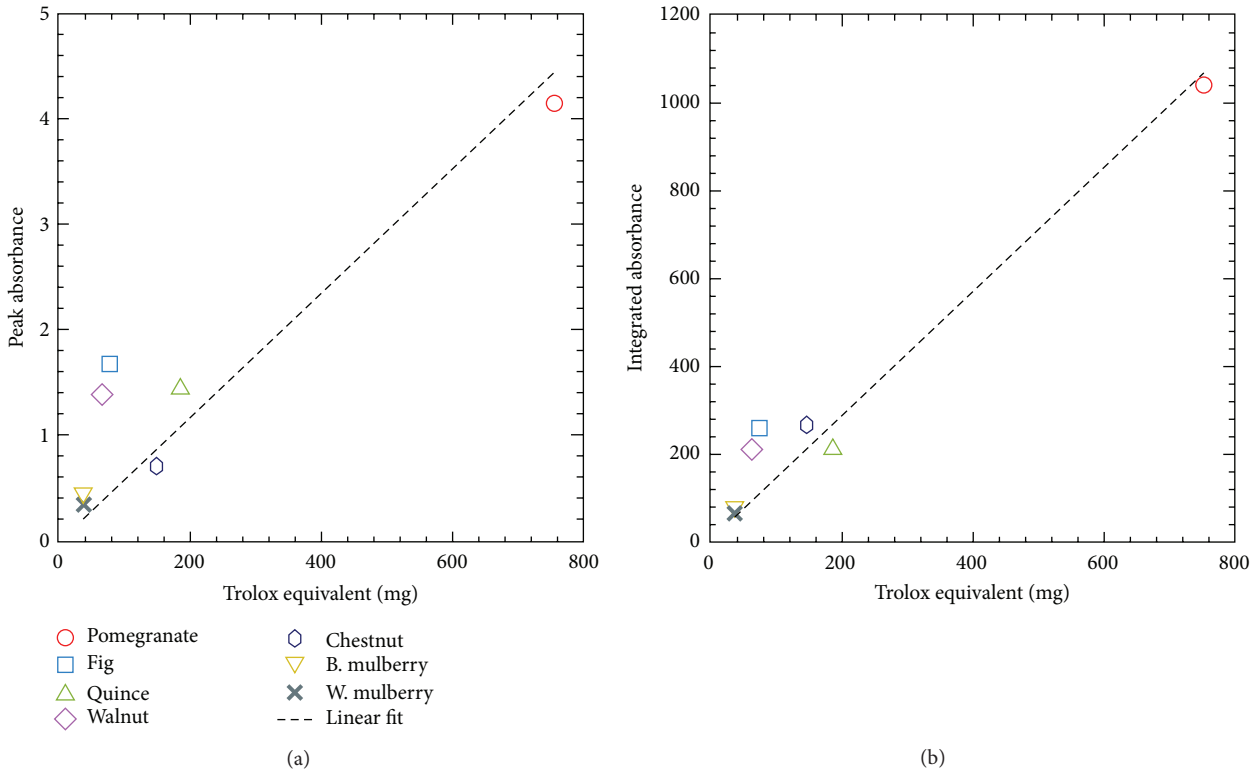


FIGURE 4: SPR peak absorbance (a) and integrated absorbance (b) between 300 and 800 nm for the mixtures in Figure 3(a) against the trolox equivalence of corresponding plant leaf extracts. Baseline subtraction is performed before the integration for data in (b). Dashed lines indicate the best fits to data.

and 800 nm recorded 120 minutes after the constituents were mixed reveals better linear correlation (Figure 4(b)) with trolox equivalence of the plant leaf extracts with a coefficient of determination (R^2) of 0.9333 than that of corresponding SPR peak absorbance with $R^2 = 0.7221$ (Figure 4(a)). Clear differences revealed by the pomegranate leaf extracts in Figures 3 and 4 are, in fact, in conjunction with the reports that pomegranate leaf exhibits higher antioxidant capacity than the similar species due to its higher total phenolic content [31, 32] which is concentrated in its leaves rather than the seeds and flowers [33].

Absorbance in the long wavelength region in Figure 3(a) may be due to increase in AgNP size, aggregation, and/or increase in shape anisotropy during the AgNP formation progress as the peak absorbance remains fixed or decreases [30]. Determining TAC of a plant leaf extract by measuring the absorbance value at a fixed wavelength and at a fixed time after mixing the constituents as in [24] can be misleading since the nucleation and the growth processes of AgNPs in the mixture of $AgNO_3$ solution and the plant leaf extracts are time-dependent processes (Figure 3(c)). Although the absorbance value at a specific wavelength, that is, at the peak wavelength, could be used as an indicator, it would be insufficient to reflect the overall phenomenon taking place over the whole wavelength range of SPR peak. Moreover, determining TAC of the plant leaf extracts using the integrated absorbance of its SPR band after its peak reaches saturation could be a better choice.

4. Conclusion

Correlation between antioxidant capacities and AgNP formation rates of pomegranate, quince, chestnut, fig, walnut, black mulberry, and white mulberry leaf extracts is investigated under an illumination of 295 lumens. Results suggest that integrated absorbance over the whole SPR band rather than its peak value should be incorporated for the total antioxidant capacity measurements in CUPRAC method. It is also pointed out that conducting antioxidant capacity measurements after the integrated absorbance over the SPR band reaches saturation would be better for the plant extracts.

Competing Interests

The authors declare that they have no competing interests.

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