Thin-Layer Densitometry as an Alternative Tool in the Quantitative Evaluation of the Free Radical Scavenging Activity of Natural Antioxidants

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An HPTLC system commonly used for chromatographic separations and analytical quality control has been utilized non-chromatographically as a screening tool for antioxidant activity in the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free-radical scavenging assay. The accurate and precise capabilities for sample application and densitometric analysis of dry spots were applied to determine the 50% inhibition of DPPH (IC_{50}) by a number of natural antioxidants at 530 nm. Concentration-response curves were generated from 3rd degree polynomial fitting of data acquired for each antioxidant at 4 concentration levels. The IC_{50} values ranged from 9.0 – 332.8 μM with quercetin being the most active and silybin the least active of the pure compounds. Three antioxidant extracts were also evaluated and grapeseed extract was the most active (IC_{50} 8.3 μg/ml). The coefficients of variation for all IC_{50} values were around 5% which indicated method reproducibility and the suitability of the used system for the intended purpose. Based on its densitometric evaluation of dry spots, the described technique provides an alternative to the spectrophotometric evaluation of samples in solution.

Key words: Densitometry, DPPH Assay, Free-Radical Scavenging, Antioxidants, HPTLC

Introduction

It is well established that oxidative stress and free radicals play a significant role in the etiology of chronic degenerative diseases such as cancer, diabetes, arthritis and cardiovascular diseases. This adds to their role in the aging process and in many other ailments [1, 2]. The search for new radical scavenging compounds has therefore become an active area of drug discovery. Compounds with antioxidant free radical scavenging activity can play a major role in reducing tissue degeneration and thus can be used as cancer chemopreventive agents or as cardiovascular and hepatoprotectants. Synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) and *t*-butylhydroquinone (TBHQ), are food preservatives that are not well accepted by the general public. Moreover, potential toxicity issues may limit their use in food products [3]. On the other hand, the plant kingdom is a rich source of natural products that possess antioxidant properties and are generally safe for human consumption. Examples of such compounds include phenolic acids, flavonoid compounds, quinones and vitamins. Many plant extracts have also exhibited marked antioxidant properties. Of these, the extracts of milk thistle, commonly known as silymarin, green tea and grapeseed are among the most frequently used antioxidants [4].

Over the last decade, numerous chemical and cell based *in vitro* assays have been developed for the discovery and evaluation of new antioxidants [1]. In general, chemical assays that screen for free radical scavenging activity are good indicators for antioxidant activity. Some of the most popular of these assays include the ferric radical antioxidant potential (FRAP) [5], the oxygen radical absorption capacity (ORAC) [6] and the 2,2-diphenylpicryl-1-hydrazyl (DPPH) [7] assays. The DPPH assay was first introduced by Brand-Williams *et al.* [7] and is based on the ability of antioxidants to reduce the stable free radical DPPH, by donating a hydrogen atom or an electron, resulting in a color transformation from purple to yellow. The reduction in the intensity of the purple color can be measured colorimetrically and related to the extent of radical scavenging. The measurement is usually performed at 515 nm in a spectrophotometer or a plate reader

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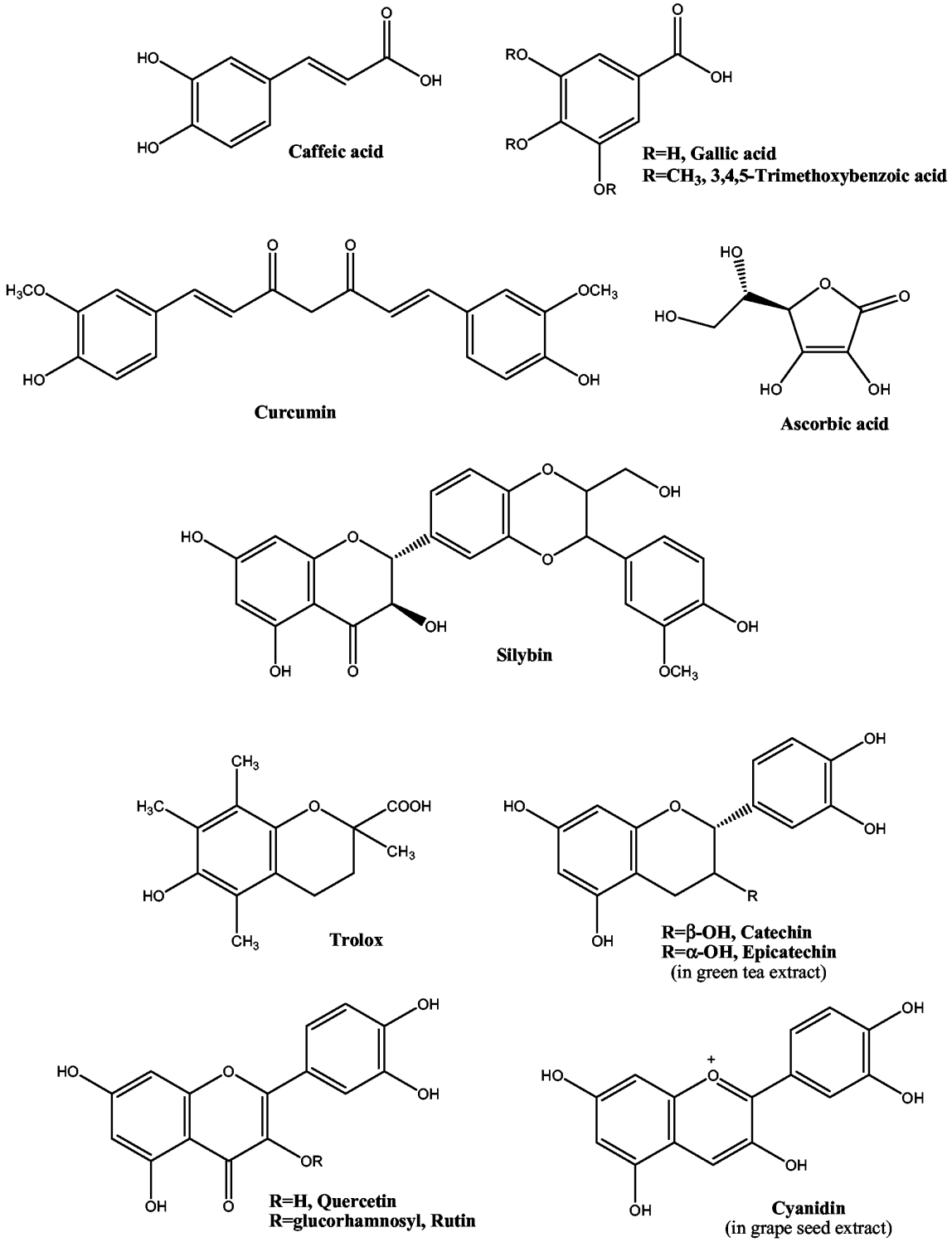

Fig. 1. Chemical structures of the pure reference compounds and the major constituents of the extracts selected for evaluation in the DPPH inhibition assay.

apparatus. Beside the classical solution-based application, the DPPH scavenging reaction has been utilized by Takamatsu *et al.* to qualitatively evaluate antioxidant activity of marine natural products applied on TLC plates. The test relied on visual examination of the color change occurring in the applied spots after spraying with a DPPH reagent [8]. Two other groups utilized TLC to detect and evaluate the antioxidant activities of phenolic constituents of different plant extracts after appropriate solvent development followed by dipping in DPPH solution [9, 10].

In this report, a quantitative method based on densitometric evaluation of dry spots is introduced as an alternative to the current absorbance based wet methods for the quantitative evaluation of DPPH scavenging activity. The method utilizes the precision capabilities of a high performance thin-layer chromatography (HPTLC) system to screen and quantify the free radical scavenging capacity of potential antioxidants without any chromatographic development and with multiple use of the same plate for more than one evaluation. As a demonstrative application of this technique, selected natural products and plant extracts (Fig. 1) with known antioxidant activity were evaluated and compared in terms of their 50% inhibitory concentration (IC_{50}) on DPPH absorbance. This application extends the utility of the HPTLC system to serve as a dual purpose unit for chromatographic analysis and for antioxidant evaluation of pure compounds and plant extracts.

Results and Discussion

A total of 13 samples known to possess antioxidant activity [8, 11–14] were selected for the assay. Ten were pure compounds and three were plant extracts (Fig. 1). Of the pure compounds, both taurine and 3,4,5-trimethoxybenzoic acid failed to inhibit DPPH at 5 mM and were not tested further. The antioxidant activity of taurine (2-aminoethane-sulfonic acid) relies primarily on mechanisms other than its weak radical scavenging activity, such as increasing glutathione levels, Fe^{3+} chelation and reduced lipid peroxidation [15, 16]. Trimethyl gallate, on the other hand, is a derivative of gallic acid with no free hydroxyl groups to participate in the DPPH radical scavenging reaction [2]. The free radical scavenging activity of all the remaining samples was concentration dependent. This was reflected in a progressive drop in the optical density of the scanned spots at 530 nm in parallel with increasing concentrations of each sample (Figs. 4 and 5, A and B). The concentration versus % inhibition relationship exhibited best fit with 3rd degree polynomial equations after non-linear regression of the data points (Fig. 5C). From the regression equations, sample concentrations causing a 50% drop in DPPH (IC_{50}) were calculated. Thus, the antioxidant activities of all tested samples could be compared (Table 1). Based on their final molar concentrations, the IC_{50}'s of all pure antioxidants were less than 40 μM except for silybin which was the least active with an IC_{50} of 332.8 μM. Quercetin was the most active compound (IC_{50} 9.0 μM). Trolox (a water-soluble vitamin E analogue) and ascorbic acid (vitamin C) exhibited nearly equal IC_{50} values. Curcumin and caffeic acid also had similar IC_{50} values and were more active than trolox and ascorbic acid. The antioxidant activity of rutin was about 50% that of its aglycone, quercetin, yet both were the most active of all the tested compounds. Grapeseed extract was the most active (IC_{50} 8.3 μg/ml) among the antioxidant extracts selected for the assay. It is also interesting that, on a weight basis, milk thistle extract (silymarin) was more active than its main component, silybin. This is probably due to participation of other flavolignan constituents of the extract, such as silychristin and silidyanin [4].

Table 1. Antioxidant activities of selected antioxidant compounds and extracts in the DPPH free-radical scavenging assay.

Compound	IC_{50} (μM ± SD, $n = 3$)[a]	(μg/ml ± SD, $n = 3$)[a]
Ascorbic acid	36.5 ±0.95	6.4 ±0.2
Trolox	35.8 ±0.4	9.0 ±0.1
Caffeic acid	25.6 ±1.9	4.6 ±0.3
Silybin	332.8 ±15.2	157.4 ±7.2
Curcumin	23.8 ±0.5	8.8 ±0.2
Rutin	15.8 ±0.8	9.6 ±0.5
Quercetin	9.0 ±0.1	3.0 ±0.03
Taurine	NA	–
Gallic Acid	14.6 ±0.7	2.8 ±0.2
3,4,5-Trimethoxybenzoic acid	NA	–
Grapeseed extract	–	8.3 ±0.3
Milk thistle extract (Silymarin)	–	61.3 ±0.5
Green tea extract	–	16.3 ±0.6

[a] Value of x determined from the regression equation $y = ax^3 + bx^2 + cx + d$ at $y = 50$; whereby a, b, c and d are the calculated coefficients. NA = not active at 5 mM.

The established use for HPTLC is in the qualitative and quantitative quality control of various entities, such as pharmaceuticals, herbal products and dietary supplements [17]. The main components of a modern HPTLC system include (i) precision sample applica-

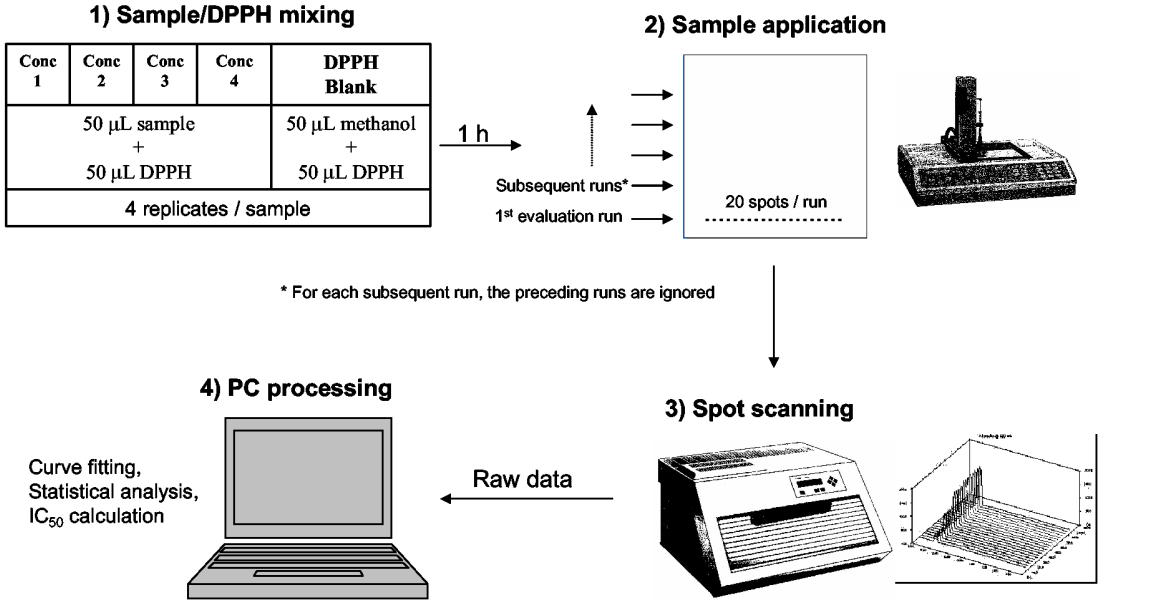

Fig. 2. Schematic representation of the overall method for antioxidant evaluation employing a combination of solution reaction and dry spot densitometric quantitation.

tor in the form of an automated micro-syringe platform; (ii) TLC plates coated with micronized adsorbent (normal or reversed-phase); (iii) solvent development chamber; and (iv) computer-controlled scanner with or without a video capture system [17]. For our purposes, the TLC plates were only used as platforms for densitometric quantitation with no subsequent solvent development of any kind. The development chamber was thus omitted. Since the DPPH assay is based on the reduction of absorbance at *ca.* 515 nm produced by the reaction of DPPH with free radical scavengers, it was plausible to investigate whether the same effect could be measured densitometrically under the current experimental setup. Preliminary UV-vis scanning of a dry spot of DPPH applied to a silica gel HPTLC plate showed a stable prominent peak at 530 nm which was close to the absorbance wavelength range (510 – 520 nm) reported in literature for DPPH solutions [12, 13, 18, 19]. Attempts to perform the reaction directly on the HPTLC plate by simultaneous application of the DPPH and trolox solutions followed by subsequent measurement of the optical density was not reproducible. On the other hand, premixing DPPH and trolox and allowing the reaction to proceed in solution followed by application and measurement on the TLC plate proved to be more accurate and reproducible. Thus, the premixing approach was adopted. Based on literature survey, the pre-measurement reaction time for wet colorimetric determination of DPPH is reported to be 2 – 30 min under different experimental settings. However, the reaction time for this method was selected to be 1 h in order to maximize response especially with slow reacting samples such as quercetin, rutin, silybin and silymarin. The band width and spacing allowed for 20 spots to be applied per run: the maximum allowed by the Linomat IV application unit. Thus, a 4-level concentration-response curve could be constructed for each sample with each level run in quadruplicate. The remaining 4 spots accommodated the DPPH blank ($DPPH_{bl}$). The sample application volume of 1 μl/spot consumed only 4 μl of sample per run and the spot drying rate of 4 sec/μl resulted in a total application time of less than 2 min/run. Fig. 2 summarizes the overall setup and evaluation procedure. One plate could accommodate at least 7 consecutive evaluations before being discarded. This could be achieved by shifting the Y application position in increments of 1 cm (1.5 cm to 7.5 cm from bottom edge) every time a new sample was evaluated. The scan range could be adjusted according to the new Y application position so that the row containing the test spots was the only one scanned per run. Alternatively, the scan range could be unchanged while discarding all the readings from previous runs. The current method

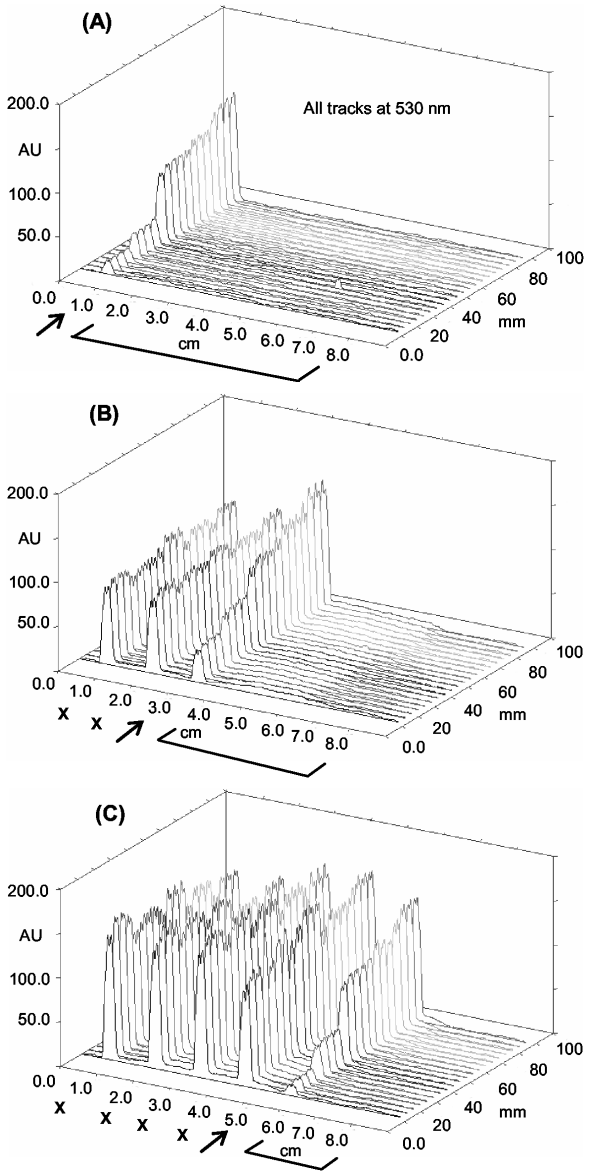

Fig. 3. Sequential evaluation of different samples on the same HPTLC plate. (A) 1st evaluation run; (B) 3rd evaluation run; (C) 5th evaluation run. (↗) new sample; (×) old evaluated samples; ([) remaining plate space for new samples. New sample spots were applied at a position 1 cm above the previously evaluated ones.

adopted the latter alternative as demonstrated in Fig. 3. Under this setup, the TLC plate is utilized in the same way as a microtiter plate with the dry spots performing the same function as the wells of the microtiter plate. The spot scanner thus performs the same function as the plate reader.

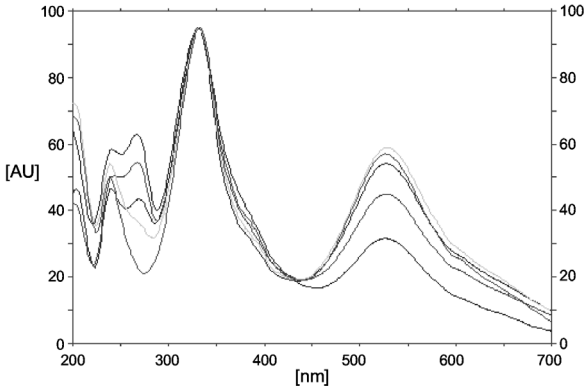

Fig. 4. UV-vis spectrum of DPPH exhibiting reduction of absorbance at 530 nm corresponding to increasing concentrations of antioxidant.

Conclusion

In addition to its established use as a chromatographic analysis instrument, the HPTLC instrument can be utilized as a screening tool for antioxidant activity employing the DPPH radical scavenging method. By the generation of concentration-response curves, the tested pure compounds could be compared in terms of their radical-scavenging activities based on their individual IC_{50} values. Plant extracts exhibiting the same activity could also be evaluated. The antioxidant data obtained under our assay settings is in line with scattered data reported elsewhere under different experimental conditions and assay setups. The introduced technique can thus be employed for the bioassay guided isolation of natural antioxidants, for SAR evaluation of closely related analogs or for selection of potential antioxidants from a group of structurally diverse compounds. The absence of any chromatographic development enhances the economical value of the assay due to the reduced consumption of TLC plates which can be used for more than one evaluation. The computer software originally designed for chromatographic applications to control sample application, spot scanning and quantification can be modified to perform all the measurements and calculations required by this assay. The need for additional 'non-native' software can thus be minimized. The current application demonstrates the versatility and adaptability of a standard HPTLC system to serve an additional purpose in the drug discovery area. Although spectrophotometers and plate readers are ubiquitously available to perform solution-based measurements in the DPPH and other assays, the proposed application

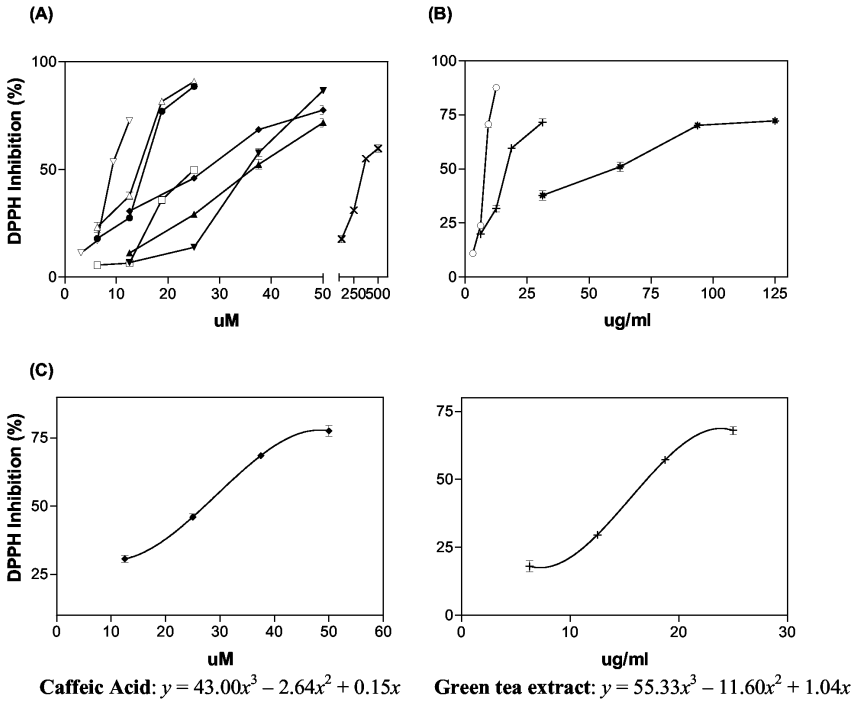

Fig. 5. Inhibition of DPPH by selected (A) antioxidants and (B) plant extracts; and (C) representative fitted curves for the calculation of IC_{50} of caffeic acid and green tea extract. Quercetin (▽), rutin (△), gallic acid (●), curcumin (□), caffeic acid (⧫), trolox (▲), ascorbic acid (▼), silybin (×), grapeseed extract (○), green tea extract (+), silymarin (∗).

provides a cost-effective alternative to the established technology.

Experimental Section

1. General. Curcumin, taurine, and dry silymarin were purchased from Sigma-Aldrich, Germany. Caffeic acid, 3,4,5-trimethoxybenzoic acid, rutin, quercetin, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (trolox) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were from Fluka, Germany. Ascorbic acid was from Alan Pharma, U.K. Grapeseed extract was from Alpha Medicine, Cairo, Egypt. Green tea extract was from Ottoman-Royal, Cairo, Egypt. Silybin was isolated by suspending silymarin in methanol, ultrasonication for 15 min and filtration. The residue was resuspended in a new batch of methanol, ultrasonicated for 15 min, and filtered. The same procedure was repeated twice on the residue to provide pure silybin (mixture of the diastereomers silybin A and B). The identity and purity of silybin was confirmed by ^{1}H and ^{13}C NMR spectroscopy. HPTLC plates (silica gel 60 F_{254}, 10 × 10 cm^2, Merck, Darmstadt, Germany) were used as supplied. Solvents for sample preparation and DPPH assay were of analytical quality.

2. HPTLC equipment. The HPTLC system (Camag, Muttenz, Switzerland) consisted of (i) TLC scanner 3 connected to a PC running WinCATS version 1.2.1 under MS Windows NT; (ii) Linomat IV sample applicator using 100 μl syringes and connected to a nitrogen tank. Plate settings: band length 2 mm, space between bands 2 mm, dosage speed 4 sec/μl; application position *y* axis: 15 mm, shifting up by 10 mm increments with every new run of the radical-scavenging assay up to 75 mm; start position *x* axis 12.0 mm. The scanner was set for maximum light optimization with slit dimension 1.20 × 1.20 micro, scanning speed 20 mm/sec, data resolution 100 μm/step. All remaining measurement parameters were left at default settings. Regression analysis and statistical data were generated by GraphPad Prism® 3.0 for Windows.

3. Free-radical scavenging assay. DPPH solution in methanol (10 mg/100 ml, 250 μM) was freshly prepared before each assay. A UV-vis spectrum of blank DPPH was initially obtained by scanning a spot of a 125 μM methanolic DPPH solution applied on an HPTLC plate. Standard methanolic solutions (100 μM) of the reference antioxidants ascorbic acid (1.76 mg/100 ml), caffeic acid (1.80 mg/100 ml) and trolox (2.50 mg/100 ml) were prepared. Each standard was serially diluted with methanol in 1 ml microfuge tubes to obtain a set of 50 μl solutions with concentrations 25, 50, 75 and 100 μM. Standard methanolic solutions (50 μM) of the reference antioxidants curcumin (1.84 mg/100 ml), 3,4,5-trimethoxybenzoic acid (0.94 mg/100 ml), quercetin (1.69 mg/100 ml) and rutin (3.05 mg/100 ml) were also prepared. Each standard was serially diluted with methanol in 1 ml microfuge tubes to obtain a set of 50 μl solutions with concentrations 12.5, 25, 37.5 and

50 μM. The starting concentration of silybin was 1000 μM (4.75 mg/10 ml) serially diluted as above to produce 250, 500, 750 and 1000 μM solutions. For the extracts, the concentration ranges were as follows: grapeseed extract 6.25, 12.5, 18.75 and 25 μg/ml; green tea extract 12.5, 25, 37.5 and 50 μg/ml; silymarin 62.5, 125, 187.5 and 250 μg/ml. Fifty μl of DPPH solution was added to each tube, gently shaken and left for 1 h at room temp. A blank control was prepared by mixing 50 μl of methanol with an equal volume of DPPH solution. After the specified time, 1 μl of each sample was applied in quadruplicate to an HPTLC plate according to the above mentioned settings. The applied 20 spots were simultaneously scanned at 530 nm and their peak areas recorded. Scanner settings are outlined above. The overall procedure is outlined in Fig. 2. For each sample, the radical scavenging activity was calculated from the equation:

$$\text{DPPH Inhibition (\%)} = \frac{[\text{DPPH}_{\text{bl}} - \text{DPPH}_{\text{test}}]}{[\text{DPPH}_{\text{bl}}]} \cdot 100$$

where, DPPH_{bl} is the peak area of the spot corresponding to blank DPPH solution and $\text{DPPH}_{\text{test}}$ is the peak area of the spot corresponding to DPPH + sample.

A curve for sample concentration versus DPPH % inhibition was plotted in Prism® by performing non-linear regression (3rd degree polynomial) on the collected data points. The 50% inhibitory concentration (IC_{50}) of each sample was calculated from the regression equation for each curve by substituting 50 for y and obtaining the unknown x in the equation $y = ax^3 + bx^2 + cx + d$, where a, b, c and d are calculated coefficients.

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