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Phytochemical, Antioxidant and Antitumor Studies of Coumarins Extracted from Granny Smith Apple Seeds by Different Methods

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ABSTRACT
The management of many human diseases linked to oxidative stress requires extensive research to explore novel agents with a potential antioxidant activity. In this study, the seeds of Granny Smith apples were extracted by four solvents; water, methanol, chloroform, and n-hexane. Extraction was performed using three techniques, which are kinetic maceration, ultrasound- and microwaves- facilitated extraction methods. These methods were carried out in three styles including non-serial, serial descendingly- and ascendingly- ordered in polarity. Phytochemical screening tests performed on the resultant extracts indicated the presence of coumarins in the methanol and chloroform extracts obtained from the applied techniques and styles. Five novel simple coumarins were isolated and their chemical structures elucidated by matching their physical properties and spectral data with those found in literature. Two in vitro biological activities were studied for the isolated products; the antioxidant strength which was studied by examining their capacity to scavenge DPPH and hydroxyl free radicals, and prefatory antitumor activity which was tested by MTT versus HeLa and MCF-7 cancer cell lines. The results showed that the isolated coumarins have promising free radicals scavenging capacity and antitumor activity with superiority to compounds **R3** and **R5**. It is concluded that there is a positive correlation between the antioxidant activity of the isolated coumarins and their antitumor activity.

Keywords: Apple, Phytochemical study, Coumarins, Antioxidant, Antitumor.

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INTRODUCTION
The theory of oxidative stress has been established in molecular biology and medicine since 1985 (1). This theory reports that oxidative stress results from the imbalance between free radicals and the body defense mechanisms. This is mainly due to the overproduction of free radicals and inadequate antioxidant capacity (2). Oxidative stress is involved in the pathology of many life-threating diseases including cancer, atherosclerosis, cerebrovascular diseases, diabetes mellitus, myocardial infraction, and osteoporosis (3). Phytochemicals with antioxidant activity are distributed in many groups of natural products, such as: polyphenols, flavonoids, hydroxycinnamic acids, proanthocyanidins, anthocyanins, lignans, stilbenes, and others (4).

For several decades, natural products and their biological activities have been a big secret that many researchers aim to unravel. Studies on natural products are increasingly found in literature; this is due to the growing global interest of using these products for improving human health (5). Nowadays, natural products play a significant role in the discovery of novel scaffolds which can be applied in the development of new drugs (6).

Apples are one of the most widely devoured fruits worldwide. Granny Smith apple is a green colored one characterized by its important nutritional value and health benefits (7). This apple cultivar contains more fibers, antioxidants, minerals, and vitamins than other cultivars of apple. Accordingly, consuming Granny Smith apple is advisable for better human health (8).

Generally, the seeds of apple have a mild harmful effect upon digestion due to the presence of a small quantity of amygdalin. The amount of this cyanogenic glycoside is relatively small (0.6 mg/g of dry seeds) and inadequate to cause poisonous effect unless an extremely high amount of seeds has been consumed (9).

Coumarins are a family of polar heterocyclic secondary metabolites which were extracted originally from plants. They have been extensively studied for their potential biological effects such as: antimicrobial, anticoagulant, antioxidant, anti-inflammatory, analgesic, antitumor, antidiabetic, and antithrombotic activities (10). In the plant kingdom, coumarins are found in various plant parts including fruits, leaves, roots, and flowers (11); also they can be detected in some extracts of plants' seeds (12).

According to the our survey performed in September 2019, there is a suitable number of research papers regarding the phytochemical analysis of Granny Smith apple. These papers studied the phytochemicals found in pomace (13), fruit flesh (14), or peel (15). The attempt to find any report recording the chemical composition of Granny Smith apple seeds was failed. This inspired the team work to start with this study.

The aim of this work is to isolate coumarins from Granny Smith apple seeds and test their in vitro antioxidant and prefatory antitumor activities. This goal was satisfied by achieving the following objectives: (I) extracting the seeds with four solvents including water, methanol, chloroform and n-hexane. Extraction was performed using three different techniques, which are kinetic maceration, ultrasound- and microwaves-facilitated extractions. With each technique, three styles were followed including non-serial, serial descendingly- and ascendingly- ordered in

polarity. (II) Subjecting the resultant extracts to qualitative phytochemical screening tests. (III) Isolating and purifying the coumarins from the elected extract. (IV) characterizing the chemical structures of isolated coumarins, and finally (V) studying their in vitro biological activities which included the antioxidant and prefatory antitumor activities.

Materials and methods

Solvents and chemicals employed in this work were bought from Tokyo Chemical Industry and Sigma-Aldrich. Silica gel (100-200 mesh size) was acquired from Sisco Research Laboratories Pvt. Ltd. MTT dye (Catalog No. 42000092-1) used in the cell viability test was purchased from Bio-World. The fruit was obtained from a public market and botanically identified by specialists from the College of Agriculture and Forestry/University of Mosul. The wavelength of maximum absorption (λ_{max}) and IR spectra of the isolated coumarins were scanned on Varian UV/ Visible and Bruker-Alpha ATR spectrophotometers. Using DMSO-d_6 as a solvent, the ^{1}H NMR and ^{13}C NMR spectra of the isolated products were recorded by Bruker Analytische Messtechnik GmbH (300 MHz).

Preparation of plant materials

The individual apple of purchased batch (145 kg) was cleaned thoroughly by tap water, followed by distilled water and cut into four slices manually. The acquired seeds were dried in shade at room temperature for 14 days, crushed by a coffee blender, and sieved providing a fine powder (263 g). This was placed in well-sealed containers and stored in refrigerator up to use in the next step (16).

Extraction

The seed powder was extracted with the following solvents: water, methanol, chloroform and n-hexane. Three techniques were applied; kinetic maceration (KM), ultrasound-facilitated extraction (UFE), and microwaves-facilitated extraction (MFE). With each technique, extraction was performed in three styles including non-serial, serial descendingly- and serial ascendingly- ordered in polarity. In serial styles, the powder was extracted with the first solvent in the order. The extract mixture was filtered and the filtrate subjected to phytochemical analysis while the residue was extracted with solvent next to the first in the applied order. These events were also applied for the third and the fourth solvents in the same manner (9).

Kinetic maceration

A mixture of the seed powder (2 g) in 20 ml extracting solvent was kinetically macerated via a shaker water bath (SWBR17 SHEL LAB shaking water bath, USA) at 30°C for 72 hrs. The mixture was filtered and the resulted solution refrigerated until be involved in the phytochemical analysis (17).

Ultrasound-facilitated extraction

A mixture of the seed powder (2 g) in 20 ml extracting solvent was sonicated in an ultrasonic water bath (40 kHz, 350 W, Power sonic410, Korea) at 30°C for 30 min. The mixture was filtered and the resulted solution stored in refrigerator until be used in the phytochemical analysis (18).

Microwave-facilitated extraction

A mixture of the seed powder (2 g) in 20 ml extracting solvent was irradiated in a domestic microwave oven (Moulinex - MW Steam 23L, MW531070, France) set at 100 W for 5 min. The mixture was filtered and the resulted solution stored in refrigerator for utilizing in the subsequent phytochemical analysis (19).

Qualitative phytochemical analysis

Thirty six extracts obtained from the priorly described extraction techniques and styles were phytochemically evaluated for the presence of flavonoids, coumarins, tannins, terpenoids, carbohydrates, alkaloids, fixed oils, emodins, anthraquinones, betacyanins, anthocyanins, phenols, proteins, amino acids, steroids, saponins, and glycosides according to the widely accepted methods reported by Harborne (20). Tables displayed the results of this analysis are attached in a Supplementary file.

Separation and isolation (9)

The chloroform extracted 200 g of seeds powder by utilizing UFE operated in a non-serial style was concentrated to dryness under reduced pressure. The mixture of crude products (8.219 g) in 82 ml of 1N NaOH was stirred at 50°C for 15 min and then filtered. The slightly yellowish filtrate was treated with 1N HCl drop by drop in an ice bath; the addition was terminated by the disappearance of the solution color. The mixture was stored in a refrigerator for 24 hr to complete the generation of crystals, which were gathered by filtration and weighted (3.143 g).

Ascending TLC technique was used to detect the number of coumarins present in the extract. A solution prepared by dissolving a small amount of crystals in 2 ml chloroform was used to create a spot on the TLC plate. A mixture of chloroform: ethyl acetate (4:1) was employed as a mobile phase and the separated spots were located via UV light (366 nm). The results of triplicate showed the presence of five spots.

The isolation of products was carried out by column chromatography utilizing mixtures of ethyl acetate: ether in a sloping ratio of 1:9 to 9:1 as eluent systems, and silica gel as a stationary phase. Five novel simple coumarins named R1-R5 were detected, each one reveled a single spot in TLC, in different eluent systems.

Physical properties and spectral elucidation of compounds R1-R5

Officinalin (R1): Off-white powder; Eluent system (3:7); Weight 0.701 g; 22.30% Yield; mp 195-198°C; R_f 0.54; UV (EtOH) λ_{max} 270 nm; IR ν_{max} 3186, 3044, 2980, 1717, 1695, 1670, 1586 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 300 MHz): δ= 11.30 (1H, s, OH-7), 8.12 (1H, s, H-5), 7.76 (1H, d, J= 9 Hz, H-4), 6.82 (1H, s, H-8), 6.24 (1H, d, J= 9 Hz, H-3), 4.10 (3H, s, H-12) ppm; ^{13}C-NMR (DMSO-d_6, 75 MHz): δ= 170.2 (C, C-

11), 165.1 (C, C-7), 160.9 (C, C-2), 160.0 (C, C-9), 143.7 (CH, C-4), 131.4 (C, C-5), 115.4 (CH, C-3), 112.7 (C, C-10), 110.9 (C, C-6), 105.6 (CH, C-8), 53.5 (CH_3, C-12) ppm.

8-(tert-butyl)officinalin (R2): White powder; Eluent system (2:8); Weight 0.212 g; 6.75% Yield; mp 202-205°C; R_f 0.77; UV (EtOH) λ_{max} 272 nm; IR ν_{max} 3187, 3046, 2982, 2933, 1717, 1694, 1669, 1585.20 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 300 MHz): δ= 11.23 (1H, s, OH-7), 8.03 (1H, s, H-5), 7.75 (1H, d, *J*= 9 Hz, H-4), 6.25 (1H, d, *J*= 9 Hz, H-3), 4.21 (3H, s, H-12), 1.5 (9H, s, H-2') ppm; ^{13}C-NMR (DMSO-d_6, 75 MHz): δ= 170.2 (C, C-11), 165.8 (C, C-7), 160.9 (C, C-2), 159.7 (C, C-9), 143.7 (CH, C-4), 129.2 (C, C-8), 128.3 (CH, C-5), 115.4 (CH, C-3), 112.4 (C, C-10), 110.4 (C, C-6), 53.5 (CH_3, C-12), 33.1 (C, C-1'), 22.1 (CH_3, C-2') ppm.

8-Hydroxyofficinalin (R3): Pale yellowish crystals; Eluent system (6:4); Weight 1.293 g; 41.14% Yield; mp 210-212°C; R_f 0.46; UV (EtOH) λ_{max} 312 nm; IR ν_{max} 3190, 3050, 2952, 1726, 1703, 1670, 1588 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 300 MHz): δ= 10.41 (1H, s, OH-8), 9.44 (1H, s, OH-7), 7.73 (1H, d, *J*= 9 Hz, H-4), 7.52 (1H, s, H-5), 6.23 (1H, d, *J*= 9 Hz, H-3), 4.19 (3H, s, H-12) ppm; ^{13}C-NMR (DMSO-d_6, 75 MHz): δ= 170.2 (C, C-11), 160.9 (C, C-2), 152.2 (C, C-7), 147.1 (C, C-9), 143.7 (CH, C-4), 134.4 (C, C-8), 124.0 (CH, C-5), 115.4 (CH, C-3), 113.1 (C, C-10), 112.2 (C, C-6), 53.5 (CH_3, C-12) ppm.

Officinalin-8-acetic acid (R4): White crystals; Eluent system (8:2); Weight 0.372 g; 11.84% Yield; mp 222-225°C; R_f 0.40; UV (EtOH) λ_{max} 276 nm; IR ν_{max} 31945, 3015, 2902, 1716, 1710, 1680, 1575 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 300 MHz): δ= 13.01 (s, 1H, OH-2'), 11.22 (1H, s, OH-7), 7.95 (1H, s, H-5), 7.75 (1H, d, *J*= 9 Hz, H-4), 6.25 (1H, d, *J*= 9 Hz, H-3), 4.11 (3H, s, H-12), 3.21 (2H, s, H-1') ppm; ^{13}C-NMR (DMSO-d_6, 75 MHz): δ= 177.1 (C, C-2'), 170.2 (C, C-11), 166.6 (C, C-7), 161.3 (C, C-9), 160.9 (C, C-2), 143.7 (CH, C-4), 130.2 (CH, C-5), 119.6 (C, C-8), 115.4 (CH, C-3), 113.1 (C, C-10), 111.3 (C, C-6), 53.5 (CH_3, C-12), 27.5 (CH_2, C-1') ppm.

8-(2'-hydroxypropan-2'-yl) officinalin (R5): White powder; Eluent system (5:5); Weight 0.462 g; 14.70% Yield; mp 206-208°C; R_f 0.51; UV (EtOH) λ_{max} 275 nm; IR ν_{max} 3366, 3183, 3044, 2981, 2932, 1719, 1693, 1666, 1585 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 300 MHz): δ=11.10 (1H, s, OH-7), 8.10 (1H, s, H-5), 7.73 (1H, d, *J*= 9 Hz, H-4), 6.24 (1H, d, *J*= 9 Hz, H-3), 4.15 (3H, s, H-12), 3.78 (1H, s, OH-2'), 1.75 (6H, s, H-1', H-3') ppm; ^{13}C-NMR (DMSO-d_6, 75 MHz): δ= 170.2 (C, C-11), 166.7 (C, C-7), 160.8 (C, C-2), 160.4 (C, C-9), 143.7 (CH, C-4), 130.8 (C, C-8), 128.7 (CH, C-5), 115.5 (CH, C-3), 112.5 (C, C-10), 110.5 (C, C-6), 62.2 (C, C-2'), 53.5 (CH_3, C-12), 33.2 (CH_3, C-1', C-3') ppm.

Antioxidant activity

The antioxidant capacity of the isolated natural coumarins (INCs) was detected by testing their efficiency to scavenge the DPPH (1,1-diphenyl-2-picryl-hydrazyl) and hydroxyl free radicals utilizing ascorbic acid as a reference compound. From a stock solution (1mM) of the INCs in methanol, six sequential diluted concentrations (200, 100, 50, 25, 12.5, 6.25 μM) were prepared. For each test, the scavenging % of the INCs was calculated according to the following formula: Scavenging activity (%) = (A_{con} – A_{sam}/ A_{con}) × 100. Where Ac is the absorbance of positive control and as is the absorbance of specimen.

The scavenging activity expressed as SC_{50}, a concentration of the sample required to scavenge 50% of the free radicals, was measured from a diagram displayed the relationship between scavenging % and log concentration of the tested compound utilizing a non-linear regression (21).

DPPH Scavenging test

The elected concentration of the sample (1.5 ml) was mixed with methanolic DPPH solution (0.5 ml, 0.1 mM). The tested mixture was coated with aluminum foil to protect from light, and incubated at room temperature (RT) for 30 min. The ability of sample to bleach the DPPH violet color was measured spectroscopically at 517 nm versus a positive control consists of DPPH (0.5 ml, 0.1 mM) and MeOH (1.5 ml) (21).

Hydroxyl radicals scavenging test

The tested mixture was prepared by the following order of addition: an elected concentration of the sample (1.5 ml), potassium phosphate buffer (2.4 ml, 200 mM, pH 7.8), $FeCl_3$ (60 μl, 1 mM), β-phenanthroline (90 μl, 1 mM), and H_2O_2 (150 μl, 170 mM). This mixture was incubated for 5 min at RT and the absorbance at 560 nm was examined versus a positive control consisting of the tested mixture but in the absence of sample (21).

Prefatory antitumor activity

For each well of a 96-well plate, 10^4 selected cancer cells were seeded and independently handled after 24 hr with twofold diluted concentrations of the isolated products starting from 200 μM and ending at 6.25 μM. Cell viability test was verified after 72 hr of handling by getting rid of the medium, supplying MTT dye (28 μl, 3.27 mM), and subsequently incubating the treated cells for 1.5 hr at 37°C. The absorbances of the treated well (At) and untreated well (Au) were detected via a microplate reader adjusted at 492 nm. The % growth inhibition of three experiments was calculated by applying the following mathematical statement: Growth inhibition (%) = (Au – At)/Au ×100 (12).

RESULTS AND DISCUSSION

The availability and historical safety of enormous number of the natural products encourage several societies to use many of these products as a part of the alternative medicine. Significant challenges confront the researchers in the field of natural products, among them are the isolation and structural elucidation of the phytochemicals. These challenges are being complicated by the attempt to correlate the presence of certain structural features with different biological activities of the isolated products (22).

Phytochemical screening study

The powder of Granny Smith apple seeds was extracted with four solvents including water, methanol, chloroform and n-hexane. Three techniques were employed in this extraction, which are KM, UFE, and MFE; for each technique, three modes were followed including non-serial, serial descendingly- and ascendingly- ordered in polarity. The resultant thirty six extracts were examined for the presence of certain primary and secondary phytochemicals. The results of the Tables attached in a Supplementary file indicated that coumarins were found in chloroform and methanol extracts which obtained from the applied extraction techniques and modes. Generally, coumarins could be extracted by more than one solvent; this is primarily due to the type of groups substituted on coumarin nucleus and the quantity of extracting solvent which considered as a huge comparing with that of extracting coumarins (23).

Separation and isolation

The presence of lactone is a key structural motif of coumarins which are hydrolyzes once attacked by a strong nucleophile like NaOH into water-soluble salts of *cis*-cinnamic acid derivatives. Acidification of these salts results in a restoration of the original coumarins (24). In the present work, this chemical theme was applied as an isolation method for coumarins. The chloroform extract from non-serial UFE was selected to isolate its coumarin ingredients. The selection was based on the absence of certain phytochemicals in this extract including flavonoids, alkaloids, tannins, and fixed oils; where their presence may interfere with the isolation process (25).

Interpretation of the chemical structures

By reviewing the literature, it was found that there is a remarkable agreement between the physical properties and spectral data of R1 and those of officinalin (26–28); thus the isolated compound titled R1 was recognized to be officinalin. Accordingly, the chemical structures of INCs, as shown in Figure 1, were elucidated depending on the chemical evidence obtained from the above accord and on the analysis of their FTIR, ^{1}H-NMR and ^{13}C-NMR spectra. The isolated coumarins are novel with an exception of compound R1.

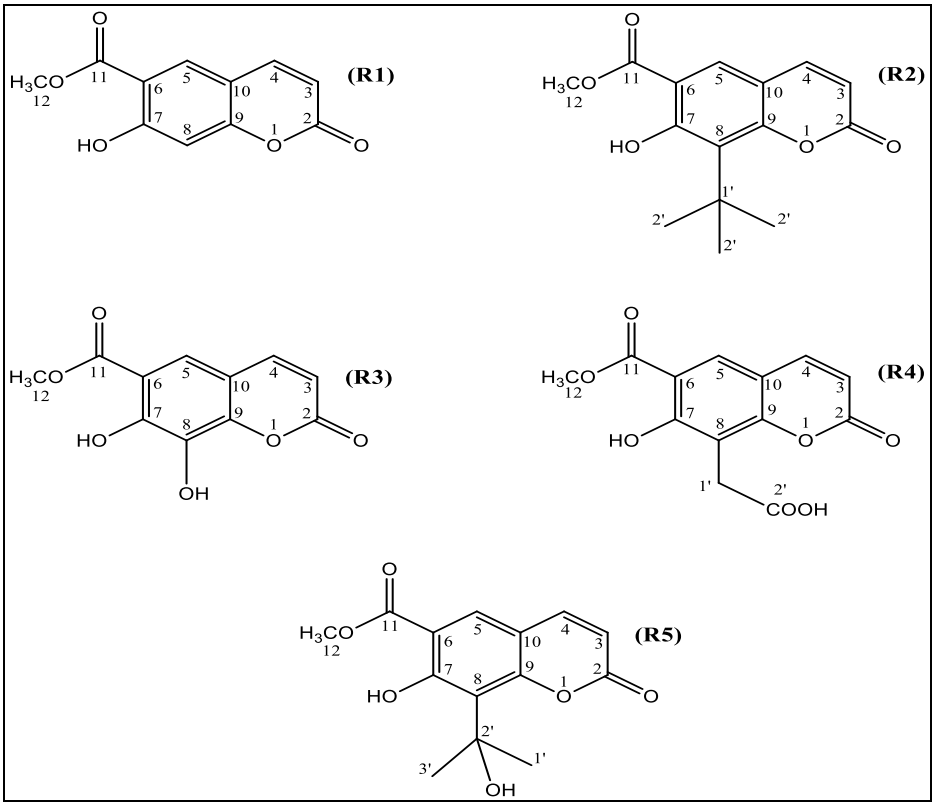

Figure 1: The chemical structures of INCs.

Antioxidant activity

In the recent years, there is a growing interest toward the antioxidant research due to its potential role on the prevention and management of many diseases affecting human health such as diabetes, cancer and Alzheimer. The significant part of this interest has focused on the finding new antioxidants isolated from natural sources (29).

In this context, a correlation between the structural features of coumarins and their antioxidant capacity has been

highlighted and frequently found in literature. This capacity may be related to the number of hydroxyl groups attached to the benzene part of coumarin nucleus (30) and to the ability of the ortho substitution to hydroxyl group for donating electrons (31). This relation is considered by observing the results shown in Table 1. Compound R3, which has two phenolic hydroxyl groups, showed a bitter ability to scavenge DPPH and hydroxyl free radicals than the other INCs. Compound R5, which has the strongest electron-donating group belongs to carbon in ortho position to phenolic hydroxyl group, showed the second best activity among the other INCs.

Table 1: Results acquired from studying the antioxidant and antitumor activities of references and INCs.

Compound name	Antioxidant activity SC_{50} (μM) ± SD (*n=3*)		Prefatory antitumor activity IC_{50} (μM) ± SD (*n=3*)	
	Scavenging of DPPH radicals	Scavenging of OH radicals	HeLa Cancer cell line	MCF-7 Cancer cell line
Reference	46.29 ± 0.67	50.33 ± 0.91	13.11 ± 0.81	12.46 ± 1.10
R1	103.09 ± 0.80	97.03 ± 0.97	104.45 ± 0.88	50.69 ± 0.78
R2	56.29 ± 0.83	58.99 ± 0.95	50.40 ± 0.76	45.44 ± 0.46
R3	48.20 ± 0.86	52.84 ± 0.76	25.11 ± 0.86	24.17 ± 0.82
R4	57.14 ± 1.05	61.39 ± 1.01	52.47 ± 0.88	45.70 ± 1.07
R5	52.21 ± 0.93	54.25 ± 0.92	25.90 ± 0.90	26.39 ± 1.08

Prefatory antitumor activity

Cell viability assay was adapted by employing MTT dye, two cancer cell lines including HeLa (cervix) and MCF-7 (breast), 5-Fluorouracil as a positive control, and DMSO as a negative control. This assay was performed to detect the prefatory antitumor activity of the INCs and to correlate their results with those acquired from testing the antioxidant activity. The data shown in Table 1 reported two issues; the first is the isolated products especially compounds R3 and R5 revealed a promising antitumor activity versus the test cell lines comparing with a positive control. The second issue is the results of antitumor activity are highly correlated with those of antioxidant activity (32–34). There is a considerable connection between oxidative stress and the propagation of tumor. Various in vitro and in vivo studies reported that the intake of exogenic antioxidants may limit free radicals damage to essential cellular components and so dropping the risk of cancer development (35). Nowadays, the concurrent administration of antitumor agents and natural antioxidants represents a strategic plan in the fighting of cancer evolution(36) .

CONCLUSION

This work demonstrated the success in the isolation and structural characterization of five novel simple coumarins from Granny Smith apple seeds. The isolated products showed hopeful antioxidant and antitumor activities with a positive correlation between them. It is proposed that the antitumor activity of the isolated coumarins may be attributed to their antioxidant capacity, and this may serve better in the design of new antioxidant and antitumor agents.

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CONFLICT OF INTEREST

There are no conflicts of interest.

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