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Comparison of Prooxidant Activities of Various Fruit Juices and Herbs via Gold Nanocluster Biosensors and Carbonyl Assay

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Abstract

Under oxidative stress conditions, which are defined as the deterioration of antioxidant and prooxidant balance in the organism in favor of prooxidants, ROS species that trigger the formation of various diseases occur. The fact that these harmful species cause oxidative damage to biological macromolecules is expressed as prooxidant activity. In this study, Cu(II)–catalyzed prooxidant activities of pomegranate, apricot, peach, and pear juices and extracts of mint, white tea, and rosehip were measured by using gold nanoclusters synthesized *via* chicken egg white proteins. Fluorometric and spectrophotometric gold nanocluster biosensors and carbonyl assay were used. The fruit juices were used directly by diluting with pure water. Herbal plant samples were extracted in an ultrasonic water bath, filtered through microfilters, and stored in the refrigerator. Total prooxidant activities of fruit juices and herbal plants were calculated in terms of mM epicatechin equivalent, and the results obtained by applying all methods were compared with each other. It has been found that the applied methods can be used to accurately determine the total prooxidant activity of many food products.

Keywords: Prooxidant activity; Gold nanocluster; Protein oxidation; Biosensor; Fruit juice; Herb.

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Çeşitli Meyve Suları ve Şifalı Bitkilerin Prooksidan Aktivitelerinin Altın Nanoküme Biyosensörleri ve Karbonil Yöntemi ile Karşılaştırılması

Öz

Organizmadaki antioksidan ve prooksidan dengesinin prooksidanlar lehine bozulması olarak tanımlanan oksidatif stres koşulları altında, çeşitli hastalıkların oluşumunu tetikleyen ROS türleri meydana gelmektedir. Bu zararlı türlerin biyolojik makromoleküllerin oksidatif hasarına sebep olması prooksidan aktivite olarak ifade edilir. Bu çalışmada yumurta akı proteinleri ile sentezlenen altın nanokümeler kullanılarak nar, kayısı, şeftali ve armut suları ile nane, beyaz çay ve kuşburnu özütlerinin Cu(II)–katalizli prooksidan aktiviteleri ölçüldü. Florometrik ve spektrofotometrik altın nanoküme biyosensörleri ile karbonil yöntemi kullanıldı. Meyve suları doğrudan saf suyla seyreltilerek kullanıldı. Şifalı bitki örnekleri ise ultrasonik su banyosunda ekstrakte edildikten sonra mikrofiltreden süzülüp buzdolabında saklandı. Meyve suları ve şifalı bitkilerin toplam prooksidan aktiviteleri mM epikateşin eşdeğeri cinsinden hesaplandı ve tüm yöntemlerin uygulanmasıyla elde edilen sonuçlar birbirleri ile karşılaştırıldı. Uygulanan yöntemlerin birçok gıda ürününün toplam prooksidan aktivitesinin hassas bir şekilde tayin edilebilmesi için kullanılabileceği görüldü.

Anahtar Kelimeler: Prooksidan aktivite; Altın nanoküme; Protein oksidasyonu; Biyosensör; Meyve suyu; Şifalı bitki.

1. Introduction

Antioxidants are substances that significantly prevent or delay the negative effects of free radicals and reactive species against biological macromolecules when they are present in foods or the body at low concentrations [1]. Prooxidants are toxic substances that induce oxidative damage in biomacromolecules and various pathological events or diseases. Oxidative stress may form as a result of the imbalance of antioxidants and prooxidants in the presence of transition metal ions present in the organism. These ions induce the redox cycling of natural antioxidants leading to the formation of reactive species such as reactive oxygen, reactive nitrogen, and phenoxy radicals that can damage cellular macromolecules such as DNA, lipids, and proteins in the presence of oxygen resulting appear chronic diseases and cancer [2]. It has recently been acknowledged that almost every disease is owing to some level of oxidative stress.

Proteins the major targets in the organism are attacked by free radicals resulting in some covalent changes directly or indirectly. Direct oxidative attacks involve transition metal ion catalyzed site-specific oxidative damage, where the reduced form of protein-bound metal ion

caused to occur hydroxyl radicals *via* Fenton reaction. Radical–directed oxidation stimulates releasing carbonyl groups in amino acids as markers of oxidative protein damage, especially in lysine, arginine, prolidine, and tyrosine [3]. Protein oxidation detection is commonly based on the measurement of stable dinitrophenyl (DNP) adduct formed from the reaction between carbonyl groups and 2,4–dinitrophenylhydrazine (DNPH) reagent [4, 5]. Nevertheless, this assay has some critical drawbacks: 1) require the use of a significant amount of decontaminated protein; 2) incapable to distinguish oxidized/nonoxidized proteins in cell or tissue; 3) non–enzymatic glycation of proteins may add carbonyl groups onto amino acid residues; 4) some plant and protein components have considerable absorbance at 370 nm such as hemoglobin and myoglobin; 5) nucleic acids in plant extracts contain carbonyl groups that can cause positive errors [3, 6, 7]. Hence, carbonyl groups must not be accepted quantitatively indicative of protein oxidation.

Pomegranate which has been used in conventional medicine for centuries in ancient cultures is a Middle Eastern fruit that extends throughout the Mediterranean region, eastward to India and China [8]. Major bioactive components of pomegranate are flavan-3-ols, flavonols, hydroxybenzoic acids, hydroxycinnamic acids, anthocyanins, gallotannins, and ellagitannins [9, 10]. Apricot fruit and products are attracted because of their special aroma, nutritive values, and taste. Major compounds of apricot are flavonoids, anthocyanins, and especially phenolic acids [11]. Peach that is native to South Asia and grown worldwide is one of the most widely consumed fruits in some European countries, especially those with the Mediterranean diet. Major compounds of peach are hydroxycinnamic acids, flavanols, anthocyanins, and flavonols [12]. Pear fruits that are widely extended throughout the temperate areas of the world, such as Australia, America, and China are in favor among consumers due to their easy digestibility and desirable taste. Major compounds of pear are phenolic acids and flavonoids [13].

Mint genres which had approximately 25–30 types distributed in Europe, Asia, Africa, North America, and Australia are widely utilized as fresh vegetable, food, herbal tea, medicine, flavoring, and spice for centuries. Major compounds of mint are phenolic acids and flavonoids. Especially the amount of rosmarinic acid in the mint extract is too high in the majority [14, 15]. White tea is originated and mainly produced in the southeastern coastal region of China and is known for its minimum processing and natural characteristics. Major compounds of white tea are phenolic acids, proanthocyanidins, glycosylated, and acetylated derivatives of flavan-3-ols and flavonols especially catechins [16, 17]. Rosehip has healing effects in inflammatory diseases, infections, flu, skincare, anti-ulcer treatments, and chronic pains. It is also used in commercial foodstuffs as a healthy ingredient in tea, marmalades, jellies, jams, soups, probiotic drinks, and

yogurts. Major compounds of rosehip are ascorbate, ß-carotene, glutathione, α-tocopherol, anthocyanins, resveratrol, phenolic acids, proanthocyanidins, flavonols, and flavanols [18, 19].

Natural antioxidants are found in abundance in cereals, fruits, herbs, and beverages. Antioxidants found in these sources are vitamins, carotenes, phenolic compounds such as tocopherols, flavonoids, phenolic acids, alkaloids, chlorophyll, and nitrogenous compounds such as protein, amine, polyfunctional organic acids. However, it has been proven that these health–beneficial compounds have prooxidant activity at high metal concentrations, high pH, and in an oxygenated environment [20-22]. Since prooxidant activity is not common unlike antioxidant capacity, its mechanism is not easily understandable and its effects are not well-known, is an area that needs more research and development. In this context, the determination of free radical formation and prooxidant activity is important in terms of applying an antioxidant-rich diet and/or drug use to reduce the risk of developing cancer and chronic diseases. Various prooxidant activity determination methods have been previously developed by our research group such as solution-based, solid protein-based, and nanocluster-based biosensors [23-28]. In this study, among these methods, gold nanocluster biosensors were applied to measure the prooxidant activities of fruit juices and plant extracts.

In the last decades, noble metal nanoclusters (NCs) have received remarkable interest due to their easy synthesis, subnanometer size, photostability, and biocompatibility [29]. These nanomaterials are particularly used for metal detection and imaging in medicine [30-32]. In this study, Cu(II)–induced total prooxidant activities of fruit juices like pomegranate, apricot, peach, and pear juices, and herbal extracts as mint, white tea, and rosehip were measured for the first time with respect to the fluorometric and spectrophotometric chicken egg white protein directed gold nanocluster (CEW–AuNC–FL and CEW–AuNC–UV) biosensors and carbonyl assay. The results obtained from all methods were compared with each other.

2. Materials and Methods

2.1. Reagents and instrumentation

The chemical substances used in this study were supplied from the corresponding sources: tetrachloroauric acid ($HAuCl_4$), 2,4–dinitrophenylhydrazine (DNPH), and neocuproine (Nc) from Aldrich (Taufkirchen, Germany); ethylenediaminetetraacetic acid (EDTA) disodium salt from Fluka (Buchs, Switzerland); sodium dihydrogen phosphate dihydrate ($NaH_2PO_4.2H_2O$), sodium hydroxide (NaOH) and ethanol (EtOH) from Sigma–Aldrich (Taufkirchen, Germany); disodium hydrogen phosphate (Na_2HPO_4), hydrochloric acid (HCl) and copper(II) sulphate from Riedel–de Haën (Seelze, Germany).

An Agilent Cary Eclipse fluorescence spectrophotometer (Santa Clara, CA, United States) was used to measure fluorescence intensity values. An Agilent Cary 100 UV–Vis spectrophotometer (Santa Clara, CA, United States) was used to measure absorbance values. A Hanna Edge pH–meter (Woonsocket, RI, United States) was used to adjust pH values of solutions using a combined glass electrode. A Select vortex apparatus was used to stir solutions. A Witeg water bath was used for the synthesis of nanocluster solution. A Bandelin Sonorex ultrasonic water bath (Berlin) was used for the extraction of herbs. A Heidolph MR Hei–Standard magnetic stirrer (Schwabach, Germany) was used to dissolve protein solution. A Shimadzu ATX222 (Kyoto, Japan) analytical balance equipped with UniBloc was used to weigh all chemicals. A Millipore Simpak1 Synergy185 (France) ultra-pure water system was used to obtain pure water.

2.2. Preparation of solutions

Neocuproine (7.5 mM) solution was prepared in EtOH. Phosphate buffer (NaH_2PO_4/Na_2HPO_4, 0.5 M, pH 7.4), 2.0 mM copper, and 0.1 M EDTA solutions were prepared in pure water. DNPH (10 mM) solution was prepared in pure water including 0.2 N HCl solution. Gold nanocluster solutions were synthesized as described in our previous work [26].

Fruit juices and herbal plants were purchased from a local market. Two grams of herb (mint, white tea, and rosehip) were weighed into a beaker, and extraction was started by adding 10 mL pure water. After 15 minutes, the supernatant was decanted, and the extraction process was repeated two times with another 10 and 5 mL pure water, respectively. The extraction process took 45 minutes in total. All extracts were passed through a GF/PET (glass fiber/polyethylene terephthalate) 1.0/0.45-μm microfilter before analysis. Fruit juices were diluted with pure water and filtered through a microfilter.

2.3. CEW–AuNC–FL biosensor

CEW–AuNC–FL was based on the quenching CEW–AuNC fluorescence intensity *via* binding Cu(I) ions to protein thiol groups (λ_{ex}=360 nm, λ_{em}=640 nm) [26]. CEW−AuNC (1 mL), 0.5 M, 0.5 mL pH 7.4 phosphate buffer, 2 mM, 0.5 mL copper(II), x mL sample solution, and (1 − x) mL of pure water were added to a test tube and incubated for 20 minutes. After adding 0.1 M, 0.5 mL EDTA solution, these mixtures were incubated for 10 minutes. Intensity values were recorded at 640 nm. Blank was prepared by adding all solutions except sample solution.

The total prooxidant activities of fruit juices and herbal extracts were calculated as mM epicatechin (ECAT) using the calibration line formed between intensity difference (ΔI) of blank

and sample, and concentration of epicatechin standard. For the CEW–AuNC–FL biosensor: ε_{ECAT} = 2860000 L mol^{-1} cm^{-1} [26].

2.4. CEW–AuNC–UV biosensor

CEW–AuNC–UV method was based on the measuring absorbance value of Cu(I)–Nc chelate formed by the reaction between protein-bound Cu(I) and neocuproine which was thought to primarily bind to the thiol groups of the protein on CEW–AuNC surface [25]. CEW−AuNC (1 mL), 0.2 M, 1 mL pH 7.4 phosphate buffer, 1 mM, 1 mL copper(II), x mL sample solution, and (2 − x) mL of pure water were added to a test tube and incubated for 30 minutes. After adding 0.5 mL, 0.1 M EDTA and 7.5 mM 1 mL Nc solutions, absorbance measurements were performed at 450 nm. Blank was prepared by adding all solutions except sample solution.

The total prooxidant activities of fruit juices and herbal extracts were calculated as mM ECAT by using the calibration line formed between absorbance and concentration of epicatechin standard. For the CEW–AuNC–UV biosensor: ε_{ECAT} = 1809 L mol^{-1} cm^{-1} [25].

2.5. Carbonyl assay

Carbonyl assay was based on the measuring absorbance values of the dinitrophenyl (DNP) hydrazone adduct formed from the reaction of the 2,4–DNPH reagent with the carbonyl groups released as a result of protein oxidation [4, 5]. CEW−AuNC (1 mL), 0.5 M, 0.5 mL pH 7.4 phosphate buffer, 2 mM, 0.5 mL copper(II), x mL sample, (1 − x) mL of pure water, and 10 mM, 1 mL DNPH solutions were added to a test tube and incubated for 30 minutes. After this period, absorbance values were recorded at 370 nm. Blank was prepared by adding all solutions except sample solution.

The total prooxidant activities of fruit juices and herbal extracts were calculated as mM ECAT by using the calibration line formed between absorbance and concentration of epicatechin standard. For the carbonyl assay: ε_{ECAT} = 6478 L mol^{-1} cm^{-1} [33].

2.6. Statistical analysis

All experiments were performed in triplicate for each sample. Statistical analyses were performed using Excel software (Microsoft Office 2016) for calculating the mean and the standard error of the mean. The significance of differences between the means of TPA values found with the CEW–AuNC–FL, CEW–AuNC–UV, and carbonyl assays was evaluated using the Pearson correlation coefficient.

3. Results and Discussion

In the last decades, nanomaterials have been utilized in various scientific and technological fields. Nanoclusters are particularly preferred in medical applications due to their very small size and not to accumulate in the body unlike nanoparticles [34]. In this study, NCs were used as a prooxidant biosensor for measuring copper-catalyzed prooxidant activities of some fruit juices and herbal extracts.

Antioxidant compounds are widely used as food ingredients or food additives [20]. Since these compounds are known to exhibit prooxidant behavior under certain conditions, the amount of dose used in the food industry becomes important. Thus, measuring prooxidant activity of phenolics known to be health−beneficial compounds has gained importance as it may guide diets carefully and in certain concentrations and prevent getting sick.

3.1. Fluorescence response to fruit juices and herbs

The specific fluorescence response of CEW–AuNC–FL at 640 nm was quenched via bonding cuprous ions to protein thiols on the NC surface. To examine the effect of fruit juices and herbs on the fluorescence response of CEW–AuNC–FL, the responses of pomegranate, apricot, peach, and pear juices and mint, white tea, and rosehip extracts in phosphate buffer medium (pH 7.4) without other solutions, i.e. nanocluster, metal ion were measured at 640 nm.

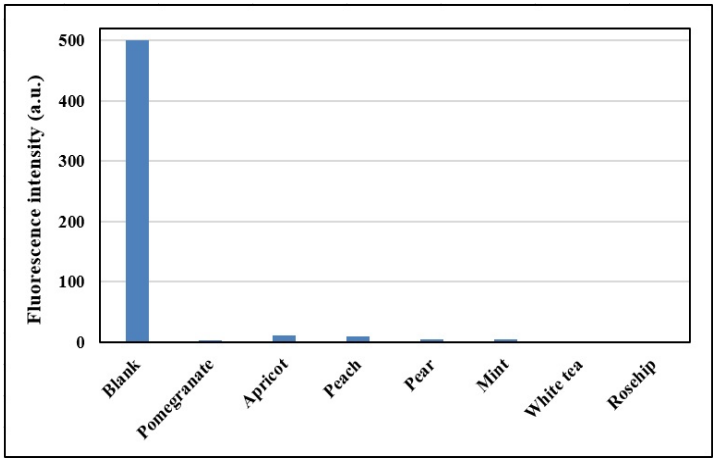

Figure 1: Fluorescence intensities of fruit juices and herbs in phosphate buffer medium (pH 7.4) at 640 nm without other solutions, i.e. nanocluster, metal ion (n=3)

The blank shown in Figure 1 was a nanocluster solution including phosphate buffer and Cu(II) solutions except for the sample. As can be seen in Figure 1, it was found that fruit juices and plant extracts did not affect the measured results as they did not have their fluorescence response.

3.2. Total prooxidant activities of pomegranate, apricot, peach, and pear juices

Total prooxidant activities (TPAs) of pomegranate, apricot, peach, and pear juices were investigated with respect to the fluorometric (CEW–AuNC–FL) and spectrophotometric (CEW–AuNC–UV) gold nanocluster biosensors and carbonyl assay. TPAs were calculated and expressed as mM ECAT equivalent by using the molar absorptivities of epicatechin given in the "Materials and Methods" section. All experiments were performed in triplicate for each sample. The results obtained can be seen in Figure 2.

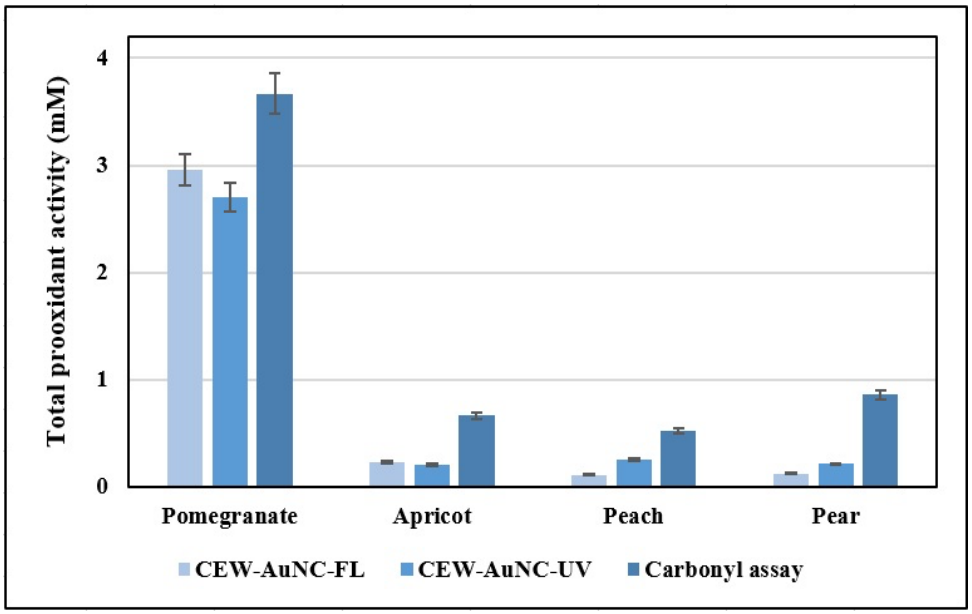

Figure 2: Total prooxidant activities of pomegranate, apricot, peach, and pear juices were calculated and expressed as mM ECAT equivalent in the bar diagram concerning the CEW–AuNC–FL, CEW–AuNC–UV, and carbonyl assays (n=3)

The TPA results obtained with AuNC biosensors were compatible unlike carbonyl assay results which were relatively higher. It is thought that the reason for the higher prooxidant activity results obtained via carbonyl assay is the presence of organic acids such as citric and malic acids, which are abundant in the content of fruits [9]. Since pomegranate fruit contains a high amount of both flavonoids and phenolic acids [10], prooxidant activity was found to be quite high compared to the other fruit juices, i.e. for pomegranate 2.96, 2.7, 3.67 mM; for apricot 0.23, 0.2, 0.66 mM; for peach 0.11, 0.25, 0.52 mM, and for pear 0.12, 0.21, 0.86 mM with respect to the CEW–AuNC–FL, CEW–AuNC–UV, and carbonyl assays respectively. The main reason why pear and apricot juice was weak prooxidant was that hydroxycinnamics were in majority compared to the flavanols in pear and apricot [35]. In the study of Slezak et al. (2017), they added high concentrations of pomegranate peels to the cell cultures and reported that pomegranate increased intracellular level

of ROS (reactive oxygen species) as behaving prooxidant activity [36]. Girard-Lalancette et al. (2009) reported that peach was slightly prooxidant at 16 μg mL^{-1} concentration on inhibiting DCFH oxidation [37]. On the other hand, no study could be found in the literature for the determination of the prooxidant activity of apricot and pear juices. In this context, prooxidant activities of related fruit juices were determined for the first time with the present study.

3.3. Total prooxidant activities of mint, white tea, and rosehip

TPAs of mint, white tea, and rosehip extracts were investigated with respect to the fluorometric (CEW–AuNC–FL) and spectrophotometric (CEW–AuNC–UV) gold nanocluster biosensors and carbonyl assay. The results were calculated as mM epicatechin equivalent using the molar absorptivities of ECAT. All experiments were performed in triplicate for each sample. The results obtained can be seen in Figure 3.

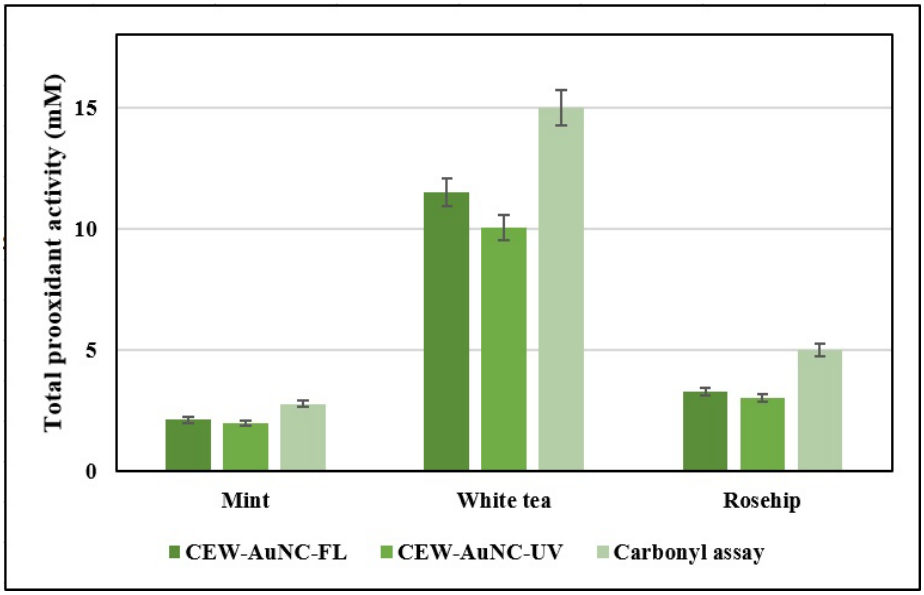

Figure 3: Total prooxidant activities of mint, white tea, and rosehip extracts were calculated and expressed as mM ECAT equivalent in the bar diagram with respect to the CEW–AuNC–FL, CEW–AuNC–UV, and carbonyl assays (n=3)

The TPA order of the herbal extracts was white tea > rosehip > mint using CEW–AuNC–FL, CEW–AuNC–UV, and carbonyl assays. This order was the same with respect to the findings of all three methods. The presence of organic acids included the carbonyl moieties in herbs cause to increase TPAs according to the carbonyl assay [19]. Therefore, carbonyl assay results were higher than nanocluster biosensors such as for mint 2.13, 2.02, 2.79 mM; for white tea 11.5, 10.06, 15.0 mM, and for rosehip 3.3, 3.05, 5.02 mM with respect to the CEW–AuNC–FL, CEW–AuNC–UV, and carbonyl assays respectively. Considering that the prooxidant activities of catechin

species are generally higher than phenolic acids, it is reasonable that the prooxidant activity of white tea rich in catechins is higher than other plants. In our previous studies, in which we used solid biosensors, the prooxidant activity of mint was found to be considerably lower than that of green tea. In these studies, the total prooxidant activity of mint was calculated as 0.50 mM ECAT equivalent according to the Cu(II)-catalyzed protein-based solid biosensor method, and 3.30 mM ECAT according to the Fe(III)-catalyzed protein-based solid biosensor method [24, 27]. Although there are many studies in the literature for the measurement of the antioxidant capacity of white tea, there is no study for determining prooxidant activity. Moldovan et al. (2016) reported that the prooxidant activity of rosehip was 6 mg mL^{-1} in tea extract [38]. Likewise, since it is known that white tea also contains catechin compounds, such as green tea, this knowledge supports the results obtained. In the study of Wang et al. (2000), it was reported that the content of flavan-3-ols found in unfermented white tea were in the following order (-)-EGCG > (-)-EGC > (-)-EC > (-)-ECG > (+)-C [39]. Also in our previous study, it was explained that especially EGCG compound had higher prooxidant activity than other catechin species [25]. Due to containing a high amount of EGCG and this compound is a stronger prooxidant than other catechins, it can be explained that white tea has much higher prooxidant activity than other herbs.

3.4. Determining significance level via Pearson correlation

The mean of total prooxidant activity values of pomegranate, apricot, peach and pear juices, and mint, white tea, and rosehip extracts measured by the CEW–AuNC–FL, CEW–AuNC–UV, and carbonyl assays were utilized to calculate significance level *via* Pearson's correlation. The Pearson correlation coefficients showed that applied methods were strongly positively correlated with each other at 95% confidence level with the values of R were 0.9998 for CEW–AuNC–FL/CEW–AuNC–UV, 0.998 for CEW–AuNC–FL/carbonyl, and 0.9979 for CEW–AuNC–UV/carbonyl (*P*–value<.00001).

4. Conclusion

In this study, total prooxidant activities of pomegranate, apricot, peach, and pear juices and mint, white tea, and rosehip herbal extracts were determined successfully with respect to the fluorometric and spectrophotometric gold nanocluster (CEW–AuNC–FL and CEW–AuNC–UV) biosensors and carbonyl assay. It was proved that the samples studied in the CEW–AuNC–FL method did not have their own fluorescence responses at the wavelength measured. Thus, it was understood that the determined prooxidant activity was only due to the fluorescence quenching of NCs with reducing the copper ion by the phenolics in the samples. The results obtained by the fluorometric and spectrophotometric CEW–AuNC biosensors were more compatible where the

carbonyl assay results were higher than the others. Since the studied samples contain high amounts of organic acid, it is thought that higher prooxidant activity was measured with positive error compared to other methods. Also, there was no significant difference at 95% confidence level *via* Pearson's correlation. The calculations showed that applied methods were strongly positively correlated with each other with the values of R were 0.9998 for CEW–AuNC–FL/CEW–AuNC–UV, 0.998 for CEW–AuNC–FL/carbonyl, and 0.9979 for CEW–AuNC–UV/carbonyl (*P*–value<.00001). The prooxidant activity of pomegranate juice was approximately 10 times higher than other fruit juices, and the prooxidant activity of white tea was approximately 5 times higher than other plant extracts. When the results of juices and herbal teas are compared, it is seen that the prooxidant activity of herbal teas is much higher. Considering these results, the consumption of herbal teas should be taken into consideration in terms of healthy life and nutrition.

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