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ORIGINAL ARTICLE

Enhanced antioxidant activity of gold nanoparticle embedded 3,6-dihydroxyflavone: a combinational study

Sharad Medhe · Prachi Bansal · Man Mohan Srivastava

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Abstract The antioxidative effect of selected dietary compounds (3,6-dihydroxyflavone, lutein and selenium methyl selenocysteine) was determined in single and combination using DPPH (2,2-diphenyl-l-picrylhydrazyl), OH (hydroxyl), H_2O_2 (hydrogen peroxide) and NO (nitric oxide) radical scavenging assays. Radical scavenging effect of the dietary phytochemicals individually are found to be in the order: ascorbic acid (standard) > lutein > 3,6-dihydroxyflavone > selenium methyl selenocysteine, at concentration 100 µg/ml, confirmed by all the four bioassays ($p < 0.05$). Among the various combinations studied, the triplet combination of 3,6-dihydroxyflavone, lutein and selenium methyl selenocysteine (1:1:1), exhibited enhancement in the target activity at same concentration level. Synthesized gold nanoparticle embedded 3,6-dihydroxyflavone further enhanced the target antioxidant activity. The combinational study including gold nanoparticle embedded 3,6-dihydroxyflavone with other native dietary nutrients showed remarkable increase in antioxidant activity at the same concentration level. The present in vitro study on combinational and nanotech enforcement of dietary phytochemicals shows the utility in the architecture of nanoparticle embedded phytoproducts having a wide range of applications in medical science.

Keywords Antioxidant · DPPH · Phytochemicals · Nanoparticle

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Introduction

Antioxidant intake has been found to be associated with lowering DNA damage, malignant transformation, cell damage and lower the incidences of certain type of degenerative diseases like cancer, heart diseases and oxidative stress (Joanne et al. 2007). There are endogenous antioxidants like glutathione and uric acid whereas most of the antioxidants are exogenous or come from our diet (Mohamad and Giridhar 2011). Epidemiological studies have highlighted the importance of consumption of dietary secondary metabolites, widely distributed in fruits and vegetables in reducing the incidence of degenerative diseases (Patricia et al. 2012). Synthetic antioxidants like butylated hydroxyl toluene, tertiary butylated hydroquinone and gallic acid esters, recently have been suspected to cause or prompt negative health effects (Anagnostopoulou et al. 2006) hence, strong restrictions are placed on their application and there is growing trend to substitute them with naturally occurring dietary antioxidants (Bera et al. 2012). Certain fruits and vegetables contain several antioxidants such as vitamins (ascorbic acid), carotenoids (lutein), polyphenols (3,6-dihydroxyflavone), and metabolic sensitizers (selenium methyl selenocysteine) better utilized to scavenge the excess free radicals from human body. Lutein is found in green leafy vegetables and prevents cellular damage by quenching singlet oxygen (Voorrips et al. 2000). The hydrophilic properties of lutein allow it to react with singlet oxygen generated in water phase more efficiently than other carotenoids (Chew et al. 2003). Its pronounced free radical scavenging ability is due to its polarity and number of conjugated double bonds. Among the dietary flavonoids, 3,6-dihydroxyflavone is ubiquitously present in the vegetables and fruits and exhibit strong antioxidant properties (Chang et al. 2010). Dietary

supplement, selenium methyl selenocysteine, exhibits significant protection against an oxidative insult (Susana 2007) and facilitates cell damaging free radical in the body (Rooseboom et al. 2002).

Researchers have achieved experimental breakthrough in the simultaneous use of two or more agents for treating any disease (Jennifer 2008). The synergistic/additive effect of various components may not only enhance the therapeutic effect but also reduce the possibilities of development of resistance (Kawabata et al. 2012). The utility of nanoparticle embedded phytoproducts has been hinted to enhance the biological activities. Considering the above facts in mind, the present paper deals with assessment of enhancement in the antioxidant activity as a result of combinational and nanotech reinforcement of dietary phytochemicals.

Materials and methods

Chemicals and reagents

All the chemicals and solvents were of AR analytical grade and purchased as follows: $NaAuCl_4$, 3,6-dihydroxyflavone and ascorbic acid (Sigma Aldrich Chemicals), selenium methyl selenocysteine (Across Organics, Belgium, Germany) and lutein (Shanghai Orgchem, China), milli-Q-water (Millipore, Bedford, A). 3,6-dihydroxyflavone, in dimethyl sulphoxide, selenium methyl selenocysteine in milli-Q-water and lutein in absolute ethyl alcohol were prepared in the concentration (100 µg/ml) and stored in small amber glass vials under Argon at −20 °C. Stock solutions were thawed and used after required dilution.

Preparation of gold nanoparticle embedded 3,6-dihydroxyflavone

In a beaker containing DMSO (5 ml), 3,6-dihydroxyflavone (5 mg) was added. The reaction mixture was stirred continuously at 25 °C for 15 min. $NaAuCl_4$ solution (100 µl; 0.1 M) was added to the stirred solution. The colour of the mixture turned light red from pale yellow within 15 min, indicating the formation of gold nanoparticles. The solution was further stirred for 45 min at 25 °C. Characterization and stability measurements were carried out using standard procedures (Katti et al. 2009).

Combinational study

In vitro antioxidant assay for each test sample, 3,6-dihydroxyflavone, gold nanoparticle embedded 3,6-dihydroxyflavone, lutein and selenium methyl selenocysteine, was carried out taking ascorbic acid as a standard. Schematic pattern of single and combinational study of test samples has been shown in Table 1.

Table 1 Schematic pattern of single and combinational study of test samples

Ascorbic acid (standard)	X						
3,6-Dihydroxyflavone		X				X	
Gold nanoparticle embedded 3,6-dihydroxyflavone			X				X
Lutein				X		X	X
Selenium methyl selenocysteine					X	X	X

Antioxidant activity

DPPH (2,2-diphenyl-1-picrylhydrazyl) assay

Free radical scavenging activity of dietary phytochemical (3,6-dihydroxyflavone), antioxidant (lutein), sensitizer (selenium methyl selenocysteine and gold nanoparticle embedded 3,6-dihydroxyflavone single and in combination) were determined using DPPH assay (Wang et al. 2008). The reaction mixture containing dilution series (10–100 µg/ml) of dietary phytochemicals were incubated with DPPH solution in MeOH (3.0 ml; 0.3 mM). The content was mixed vigorously and allowed to stand for 30 min at room temperature. The absorbance was measured at 517 nm. The lower absorbance represents the higher DPPH scavenging activity. Ascorbic acid was used as the reference antioxidant. The percent inhibition of DPPH was calculated using the formula, $[(C–T/C)] \times 100$, where C is absorbance of the control and T for the test samples.

Hydroxyl radical assay (FENTON)

The hydroxyl radical scavenging activity of selected dietary phytochemicals was determined in single and combination using FENTON reaction (Yang and Guo 2001). The reaction mixture containing dilution series from 10 to 100 µg/ml of dietary phytochemicals were incubated with deoxyribose (3.75 mM), H_2O_2 (1 mM), $FeCl_3$ (100 µM) in phosphate buffer (pH 7.4). The reaction is terminated by thiobarbituric acid (1 ml; 1 % w/v) and trichloroacetic acid (1 ml; 2 % w/v) and then heated in boiling water bath for 15 min. The pink chromogen formed eventually resulted in the formation of thiobarbituric acid reactive substances (TBARS). The content was cooled and absorbance of mixture was measured at 535 nm against blank. The percent inhibition of hydroxyl radical generation was calculated using the formula, $[(C–T/C)] \times 100$, where C is absorbance of the control and T for the test samples.

H_2O_2 scavenging activity

H_2O_2 scavenging ability of phytochemicals under study was determined (Nabavi et al. 2008). H_2O_2 solution (40 mM) was prepared in phosphate buffer (pH 7.4). The methanolic solutions of phytochemicals (10–100 µg/ml) concentration in phosphate buffer (3.4 ml) were added to H_2O_2 solution (0.6 ml; 40 mM). The absorbance of the reaction mixture was recorded at 230 nm. Blank solution contained the phosphate buffer without H_2O_2. The absorbance of all the compounds were measured at 230 nm. The percent inhibition of H_2O_2 radical generation was calculated using the formula, $[(C–T/C)] \times 100$, where C is absorbance of the control and T for the test samples.

Nitric oxide scavenging activity

Sodium nitroprusside (2 ml; 10 mM) in phosphate buffer saline was incubated with the test samples at 10–100 µg/ml concentration at room temperature for 30 min. After 30 min, incubated solution (0.5 ml) was added with Griess reagent (1 ml) and absorbance was measured at 546 nm (Ebrahimzadeh et al. 2009). The percent inhibition of NO· generation was calculated using the formula, $[(C–T/C)] \times 100$, where C is absorbance of the control and T for the test samples.

Statistical analysis

Data are expressed in percent inhibition with respect to control. All the tests were performed in triplicates and data expressed are as mean ± SD. The statistical analysis was carried out using the one-way analysis of variances (ANOVA). Post Dunnett test was applied between control, reference drug and test samples using Graph Pad Prism software. A value of $p < 0.05$ was considered for statistical significance.

Discussion

Characterization of gold nanoparticle embedded 3,6-dihydroxyflavone

Ultraviolet–Visible analysis

The formation and stability of metal nanoparticles have been ascertained by UV method (Khoee and Rahmatolahzadeh 2012). The change in colour of the solution (yellow to light red) by the addition of $NaAuCl_4$ (100 µl; 0.1 M), producing a broad peak in the range of 425–520 nm, indicates the particles are mono dispersed. The appearance of light-red colour is attributed to surface plasmon resonance arising

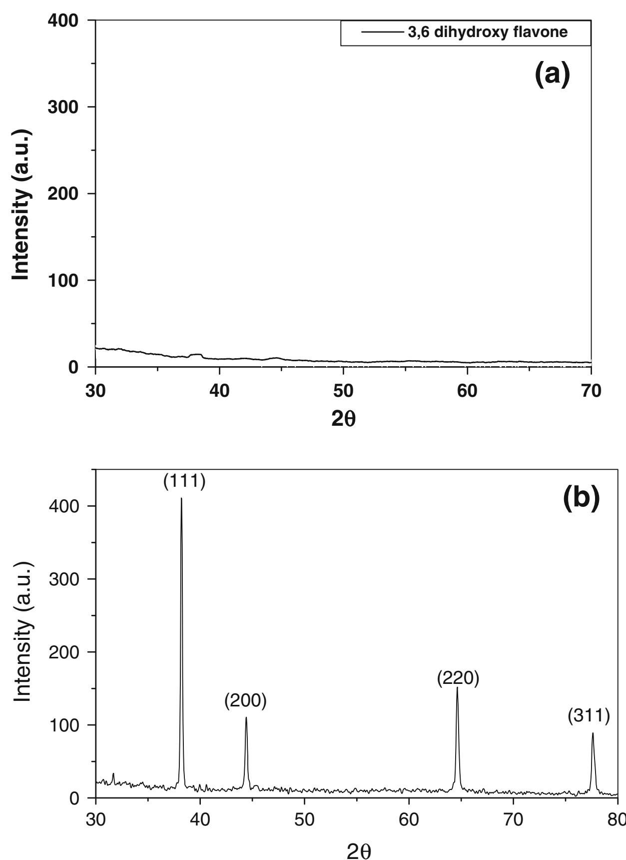

Fig. 1 **a** XRD of 3,6-dihydroxyflavone. **b** XRD of gold nanoparticle embedded 3,6-dihydroxyflavone

from free conduction electrons induced by an interacting electromagnetic field (Song and Kim 2008) and excitation of surface plasmon vibrations in the structure of 3,6-dihydroxyflavone-embedded gold nanoparticles.

XRD analysis

The XRD pattern for 3,6-dihydroxyflavone does not show any characteristic peak due to its amorphous nature while in case of gold nanoparticle embedded 3,6-dihydroxyflavone, three characteristic peaks correspond to 111, 200 and 220 of Au located at $2\theta = 38.29°$, 44.43° and 64.68°, respectively, were observed, confirming that the sample is composed of crystalline gold phase. Using Scherer's equation, $t = k\lambda/B \cos\theta$ where t is crystallite size, B is full width at half maxima, k is shape factor (0.9 for spherical particles) and λ is incident wavelength of X-ray (1.548 Å), average particle size of synthesized material was 12 nm (Fig. 1a, b).

SEM, TEM and AFM analysis

SEM micrographs of 3,6-dihydroxyflavone and gold nanoparticle embedded 3,6-dihydroxyflavone were recorded. 3,6-Dihydroxyflavone shows larger size of aggregated rhombic

crystal while uniform needle-type morphology was observed in case of gold nanoparticle embedded flavonoid is due to low temperature, polarity of solvent during synthesizing the compound and characteristic (111) peak observed in XRD pattern (Fig. 2a, b).

TEM micrographs of 3,6-dihydroxyflavone were found to be of larger size, irregular-shape while gold nanoparticle embedded 3,6-dihydroxyflavone of nucleated cell type of morphology with 6–12 nm particle size (Fig. 3a, b). EDAX spectrometry further confirmed the presence of gold in the synthesized material with no other contaminants. The appearance of optical adsorption peak at approximately 2.30 keV is the characteristic for the adsorption of gold nanocrystallites. The current profile of EDAX of gold nanoparticle embedded 3,6-dihydroxyflavone showed strong gold atom signals around 2.30, 9, 10.30, 11.30, and 12.30 keV. Our experimental findings are in the harmony of the earlier reports on the preparation of gold embedded extract particles from the plant products (Huang et al. 2007; Parashar et al. 2009; Tamizhamudu and Kantha 2011).

AFM image of native and gold nanoparticle embedded 3,6-dihydroxyflavone were recorded in plain and 2D view. Native 3,6-dihydroxyflavone shows rough surface with larger particle size while, gold nanoparticle embedded 3,6-dihydroxyflavone shows continuous and uniformly distributed particle with small size (Fig. 4a, b).

In vitro stability study

The most important criteria for molecular-imaging applications are the stability of gold nanoparticles over a reasonable time period. Therefore, the stability of gold nanoparticle

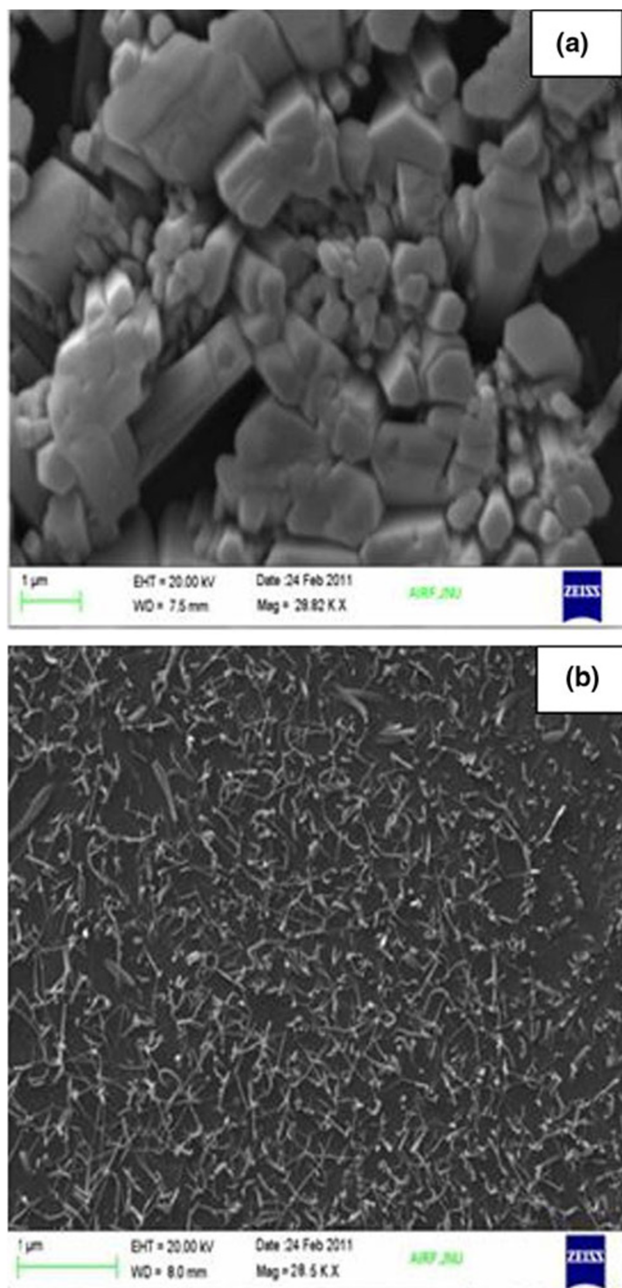

Fig. 2 **a** SEM image of 3,6-dihydroxyflavone. **b** SEM image of gold nanoparticle embedded 3,6-dihydroxyflavone

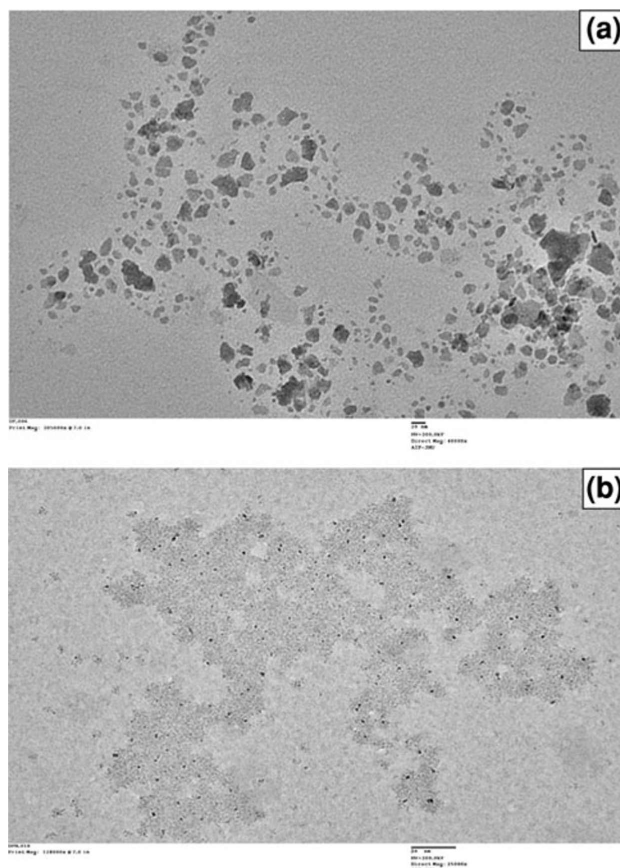

Fig. 3 **a** TEM image of 3,6-dihydroxyflavone. **b** TEM image of gold nanoparticle embedded 3,6-dihydroxyflavone

embedded 3,6-dihydroxyflavone was evaluated by monitoring the plasmon (λ_{max}) in cysteine (0.5 %), histidine (0.2 M), human serum albumin (0.5 %), bovine serum albumin (0.5 %) solutions over 30 min. The stability of gold nanoparticle embedded flavonoid was also monitored at pH 5, 7 and 9 of phosphate buffer. The plasmon wavelength in all the above formulations showed minimal shifts of approximately 1–5 nm, thereby confirming that the gold nanoparticles are intact, showing excellent in vitro stability in biological fluids at physiological pH (Chanda et al. 2010).

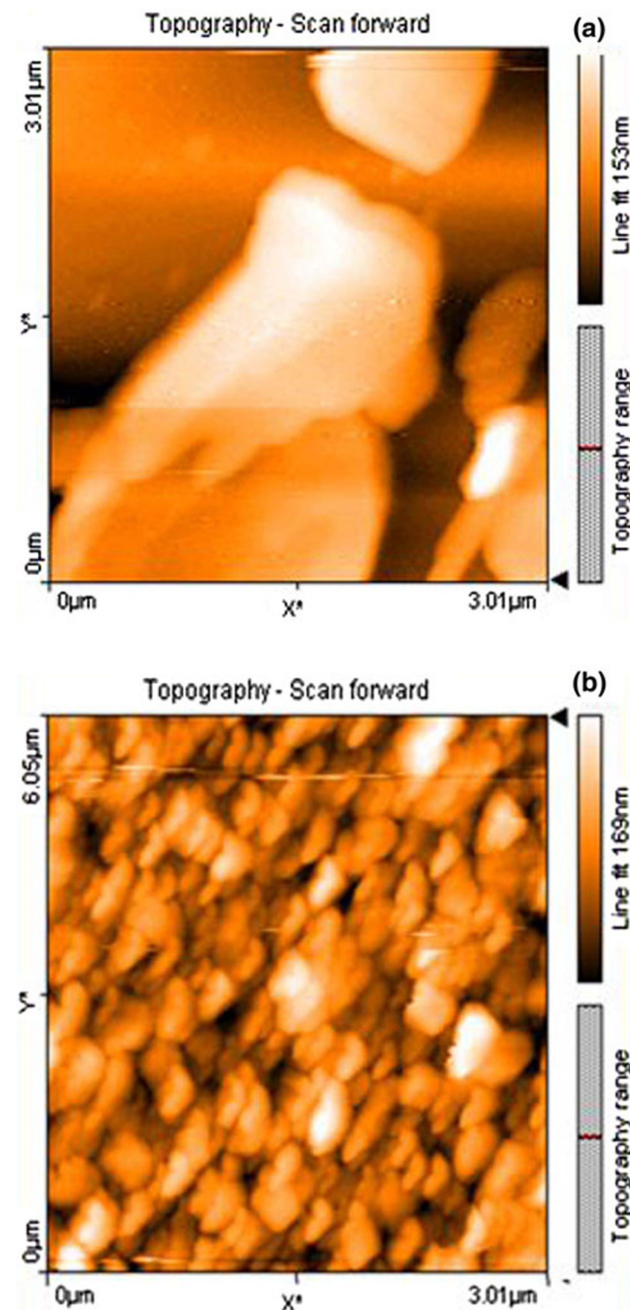

Fig. 4 **a** AFM image of 3,6-dihydroxyflavone. **b** AFM image of gold nanoparticle embedded 3,6-dihydroxyflavone (2D)

DPPH radical scavenging activity

DPPH is a stable nitrogen-centered free radical, the color of which changes from violet to yellow upon the reduction by either the process of hydrogen or electron donation. Substances which are able to perform this reaction can be considered as antioxidants and, therefore, radical scavengers (Dehpour et al. 2009). Radical scavenging activities of all the dietary compounds under study increased with increasing concentration exhibiting its dose dependant nature. Percent of inhibition for DPPH radical scavenging activity is presented in Fig. 5a. A perusal of the data shows that at concentration 100 µg/ml, all three dietary compounds individually exhibited maximum percent of inhibition: 3,6-dihydroxyflavone (64.21 %), lutein (65.79 %) and selenium methyl selenocysteine (43.85 %) against ascorbic acid (96.28 %) as standard. The triplet combination (1:1:1) exhibited maximum percent of inhibition (72.89 %) at same concentration level, indicating 14.94 % enhancement in antioxidant activity. Gold nanoparticle embedded 3,6-dihydroxyflavone individually showed maximum percent of inhibition (72.04 %) at the same concentration compared to native 3,6-dihydroxyflavone (64.21 %).The inclusion of gold nanoparticle embedded 3,6-dihydroxyflavone with other dietary nutrients lutein and selenium methyl selenocysteine further increased maximum inhibition (87.13 %) at the same concentration of 100 µg/ml. Thus, over all enhancement is obtained in the antioxidant activity (29.23 %) of triplet combination involving gold nanoparticle embedded 3,6-dihydroxyflavone.

OH radical scavenging activity (FENTON assay)

The hydroxyl radical formed in the FENTON reaction in the presence of reduced transition metals such as Fe^{2+} and H_2O_2 which are known to be the most reactive of all the reduced forms of dioxygen is capable of damaging of almost every molecule found in living cells (Rollet-Labelle et al. 1998). Deoxyribose was oxidized when exposed to hydroxyl radicals generated by Fenton reagent and the oxidation degradation can be detected by heating the products with TBA. The hydroxyl radical scavenging activity of all the dietary compounds in single and combination was determined. Increased radical scavenging activities with increasing concentration of the dietary compounds under study were noticed. Percent of inhibition for OH radical scavenging activity are tabulated in Fig. 5b. Data indicate that three dietary compounds individually exhibited maximum percent of inhibition: 3,6-dihydroxyflavone (62.11 %), lutein (63.85 %) and selenium methyl selenocysteine (41.62 %) against ascorbic acid (96.18 %) as standard at concentration 100 µg/ml. The triplet combination (1:1:1), exhibited maximum percent of inhibition

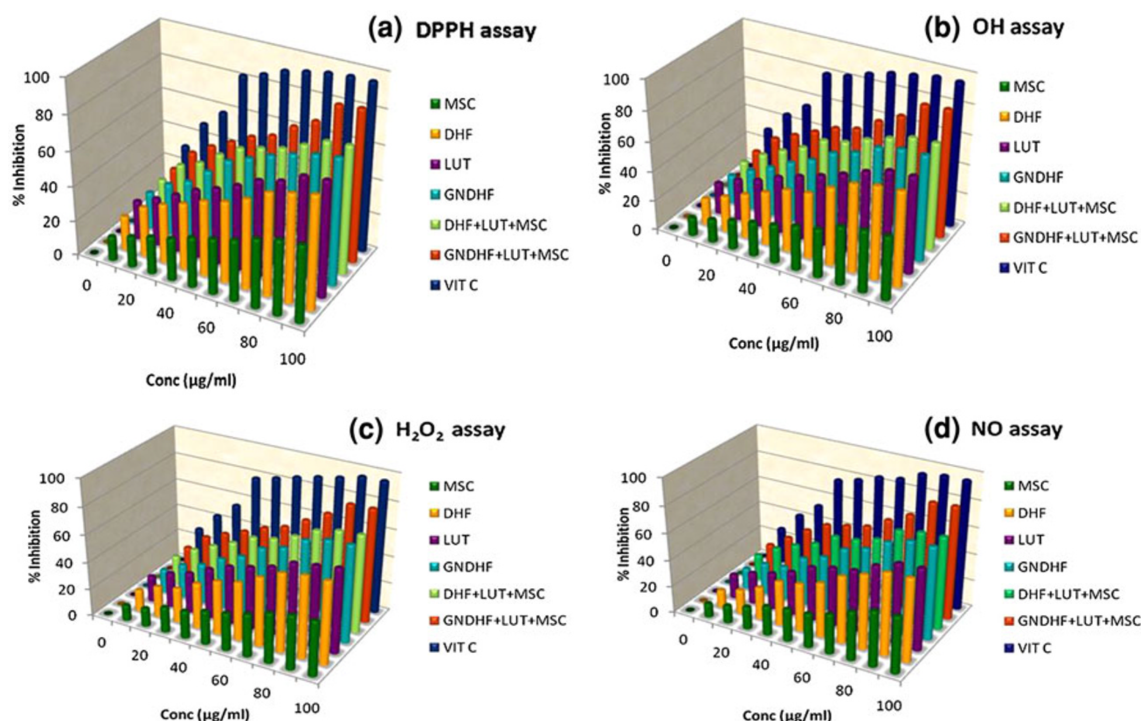

Fig. 5 **a** Percentage inhibition of DPPH radicals, **b** OH radicals **c** H_2O_2 radicals and **d** NO radicals concentration dependency of dietary compounds, single and in combination against standard ascorbic acid. Each value is mean ± SD ($n = 3$). $P < 0.05$ (single and combination) versus ascorbic acid *VIT* C ascorbic acid, *DHF* 3,6-dihydroxyflavone, *GNDHF* gold nanoparticle embedded 3,6-dihydroxyflavone, *LUT* lutein, *MSC* selenium methyl selenocysteine

(70.63 %) at same concentration of 100 μg/ml indicating (14.77 %) enhancement in antioxidant activity. Gold nanoparticle embedded 3,6-dihydroxyflavone individually showed maximum percent of inhibition (70.01 %) at the same concentration compared to the native 3,6-dihydroxyflavone (62.11 %). The inclusion of gold nanoparticle embedded 3,6-dihydroxyflavone with other dietary nutrients lutein and selenium methyl selenocysteine further increased the maximum inhibition (85.11 %) at the same concentration, with overall enhancement (26.61 %) in antioxidant activity.

Hydrogen peroxide scavenging activity

H_2O_2 scavenging by dietary compounds may be attributed to donate electrons to H_2O_2, thus neutralizing it to water. The ability of these compounds effectively scavenges hydrogen peroxide (Sroka and Cisowski 2003). Radical scavenging activities of all the dietary compounds studied increased with increasing concentration. Percent of inhibition for H_2O_2 radical scavenging activity are shown in Fig. 5c. The data shows that the dietary compounds individually exhibited maximum percent of inhibition; 3,6-Dihydroxyflavone (60.11 %), lutein (61.85 %) and selenium methyl selenocysteine (40.02 %) against ascorbic acid (96.12 %) as standard at concentration 100 μg/ml. Among the combinations studied, the triplet combination (1:1:1), exhibited maximum percent of inhibition (71.35 %) at the same concentration level, indicating 17.35 % enhancement in antioxidant activity. Gold nanoparticle embedded 3,6-Dihydroxyflavone individually showed maximum percent of inhibition (71.08 %) compared to normal 3,6-Dihydroxyflavone (60.11 %) at the same concentration level. The combinational study with the inclusion of gold nanoparticle embedded 3,6-Dihydroxyflavone with other dietary nutrients lutein and selenium methyl selenocysteine further increased maximum inhibition (83.10 %) at the same concentration. Thus, the overall enhancement could be gained in the antioxidant activity (25.45 %) of triplet combination having of gold nanoparticle embedded 3,6-dihydroxyflavone.

Nitric oxide scavenging activity

The percent inhibitions for NO radical scavenging activity are reported in Fig. 5d. A perusal of the data shows that at concentration 100 μg/ml, the three dietary compounds individually exhibited maximum percent of inhibition; 3,6-dihydroxyflavone (61.24 %), lutein (60.85 %) and selenium methyl selenocysteine (42.11 %) against ascorbic acid (96.02 %) as standard. The triplet combination (1:1:1), exhibited maximum percent of inhibition (69.09 %) at

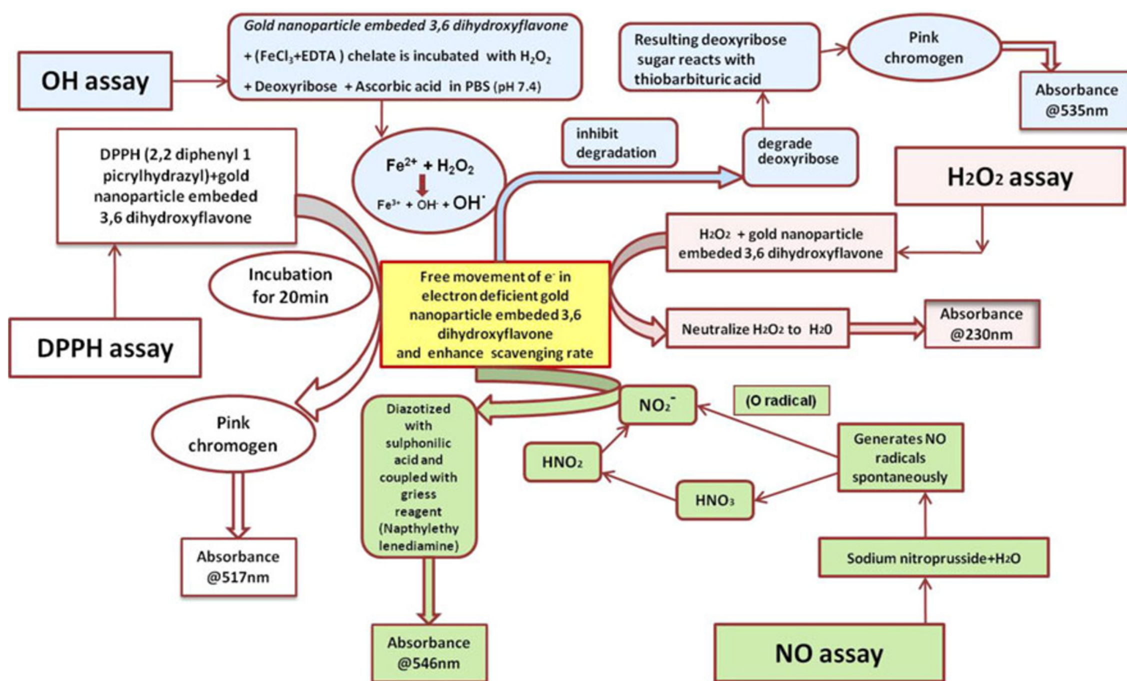

Fig. 6 Mechanism of antioxidant activity of gold nanoparticle embedded 3,6-dihydroxyflavone

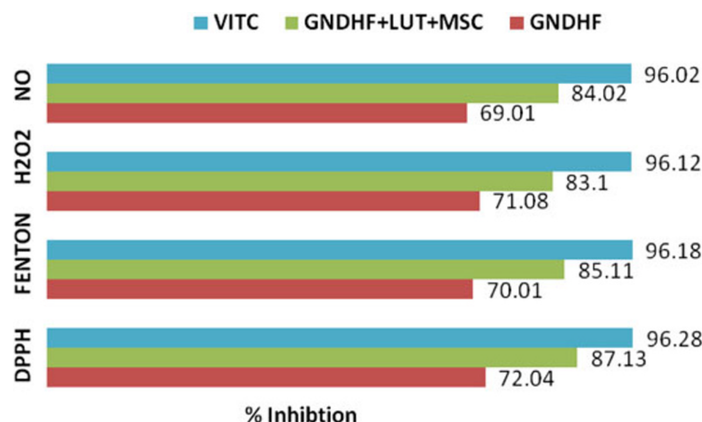

Fig. 7 Percentage inhibition of DPPH, OH, H_2O_2 and NO radical concentration of gold nanoparticle embedded 3,6-dihydroxyflavone and combination of gold nanoparticle embedded 3,6-dihydroxyflavone with lutein and selenium methyl selenocysteine against standard ascorbic acid. Each value is mean ± SD ($n = 3$). $P < 0.05$ (single and combination) versus ascorbic acid (Vitamin C) *VIT* C ascorbic acid, *GNDHF* gold nanoparticle embedded 3,6-dihydroxyflavone, *LUT* lutein, *MSC* selenium methyl selenocysteine

same concentration indicating 14.35 % enhancement in antioxidant activity. Gold nanoparticle embedded 3,6-dihydroxyflavone individually showed maximum percent of inhibition (69.01 %) at the same concentration compared to percent inhibition of normal 3,6-dihydroxyflavone (61.24 %). The inclusion of gold nanoparticle embedded 3,6-dihydroxyflavone with other dietary nutrients further increased maximum inhibition (84.02 %) at the same concentration level, exhibiting over all enhancement (Fig. 6) in the antioxidant activity (26.07 %).

The powerful antioxidant characteristic of dietary phytochemicals prompted us to test their efficacy in reducing sodium tetrachloroaurate to corresponding gold nanoparticles. We believe that effective utilization of various dietary phytochemicals that contain functional groups like hydroxyl, phenols and aromatic ring will provide synergistic power for the reduction of gold salts into their corresponding gold nanoparticle. The combination involving gold nanoparticle embedded 3,6-dihydroxyflavone with lutein and selenium methyl selenocysteine (1:1:1) (Fig. 7)

is found to be most effective for the enhancement in the antioxidant activity. Further, the coating of phytochemical on the gold nanoparticles, thus, paves an unprecedented process for the production and stabilization of gold nanoparticles simultaneously enhancing bioactivity in a singular green process. In each case, the role of gold salt solution toward antioxidant activity has been checked separately and has not been found to reflect any noticeable antioxidant activity.

Conclusions

We herein, report a green synthetic route that involves the production of well-defined spherical gold nanoparticle by simply mixing of flavonoid to an aqueous solution of sodium tetrachloroaurate. Production (within 15 min) and stabilization of gold nanoparticle is achieved using flavonoid-mediated green nanotechnological process. The studies reported in this paper provide the power of plant sciences to bring about a paradigm shifts on future development of nanotechnology. The versatile phytochemical-mediated green nanotechnological process has been shown to be effective in both, the generation and stabilization of non-toxic gold nanoparticle for direct applications in therapeutic efficiency.

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