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Invitro free radical scavenging activity and bioavailability of dietary compounds caffeine, caffeic acid and their combination

Nadanasabapathi, S., Rufia. J. and *Manju, V.

Department of Biochemistry, Periyar University, Periyar Palkalai Nagar, Salem-636 011, Tamil Nadu, India

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Abstract

A free radical scavenging activity is an important property of natural dietary compounds, caffeine and caffeic acid compound widely present in many fruits, vegetables and coffee. The present study focused on determining the antioxidant activity of caffeine, caffeic acid and (caffeine + caffeic acid) through *invitro* antioxidant experiments. *Invitro* lipid peroxidtion, 1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenging, superoxide scavenging, hydrogen peroxide scavenging, reducing power assay and total antioxidant properties. Compounds bio availability scores and structures designed by using molinspiration-cheminformatics tool and their combination (caffeine + caffeic acid) has possessed the significant free radical scavenging activity and lipid peroxidation effects in all tested methods were compared with standard antioxidant ascorbic acid. A score of bioavailability and drug likeness properties of caffeine and caffeic acid has a good score individually and the combination. Our results indicate that combination having antioxidant properties and could be used in the treatment and management of free radical mediated disease.

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Introduction

Majority of the diseases are mainly linked to oxidative stress due to free radicals. Free radicals are fundamental to any biochemical process and normal metabolism (Fang *et al.*, 2002). Reactive oxygen species (ROS) is producing during normal physiologic events and they can easily initiate the peroxidation of membrane lipids, leading to the accumulation of lipid peroxides. Under pathological conditions, ROS is overproduced and results in oxidative stress. The imbalance between Reactive oxygen species and antioxidant defense mechanisms leads to oxidative modification in cellular membrane or intracellular Molecules (Duh *et al.*, 1999). Antioxidant systems either prevent these reactive species from being formed, or remove them before they can damage vital components of the cell. Antioxidant supplements or foods containing antioxidant can interfere with oxidation process used to reduce oxidative damage in human body (Miller *et al.*, 1995; Gülçin *et al.*, 2002). Natural antioxidants can protect the human body from free radicals and retard the progress of many chronic diseases as well as retard lipid oxidative rancidity in foods (Pryor *et al.*, 1991; Lai *et al.*, 2001). Recently, interest has increased considerably in finding naturally occurring antioxidant for use in foods or medicinal materials to replace synthetic antioxidants (Zheng and Wang, 2001).

Caffeine is a widely drinking psychoactive substance present in coffee and tea. It acts a central nervous stimulant and resorting alertness (Lovett and Richard, 2005). Combined chemotherapy with caffeine is effective against tumors and enhances the anticancer drugs such as cisplain (Van Den Berg and Roberts, 1975), doxorubicin, cyclophosphamide, mitomycin C, vincristine and methotrexate (Tomita and Tsuchiya, 1989). Caffeic acid (3, 4-dihydroxycinnamic acid) is one of the major metabolites produced by the hydrolyzation of chlorogenic acid, a major phenolic phytochemical in various foods, vegetables including coffee (Nam Joo Kang *et al*., 2009). Recent research, caffeic acid was a superior antioxidant compared with p-coumaric and ferulic acids, in inhibiting LDL oxidation (Meyer *et al*., 1998). Caffeic acid has been reported to decrease the risk of chronic diseases such as inflammation, cardiovascular disease and cancer (Bonita *et al*., 2007). Caffeic acid has been shown to confer a wide spectrum of biological effects such as antioxidant, antitumor and anti-metastatic (Chung *et al*., 2004). The consumption of caffeine and caffeic acid was so large, because they are present in the most popular drink coffee in worldwide (Boyer *et al*., 2004), several experimental and clinical trials reported consumption of coffee to be positively correlated with reduced risk of human chronic diseases such as inflammation, diabetes and cardiovascular disease (Watanabe *et al*., 2006). Free radical scavenging activity of caffeine and caffeic acid combinational effects of this regimen

*Corresponding author.
Email: *manjucb11@gmail.com*
Tel: +91 9940247576; Fax: 0427 2345124

not been investigated previously.

The objectives of the present study were to investigate the invitro antioxidant activity of caffeine, caffeic acid and (caffeine + caffeic acid) their effectiveness and changes through the hydrogen donor activity, superoxide scavenging, reducing ability, hydrogen peroxide scavenging, and total antioxidant activities. Combination of these compounds structures was designed and bioavailability was predicted by using pub chem database and cheminformatics tools. There is increasing interest in collecting and applying chemical information is new trend, in which biological information and chemical information are integrated on the genome scale. (Lipinski and Hopkins, 2004).

Materials and Methods

Reagents and chemicals

Caffeine, caffeic acid, ascorbic acid, 1, 1-Diphenly-2-picryl-hydrazil (DPPH), was purchased from Sigma chemical company. nicotinamide adenine dinucleotide (NADH), potassium ferricyanide, trichloroacetic acid (TCA) nitroblue tetrazolium (NBT) and phenazine methosulphate (PMS), DMSO was purchased from Himedia chemical company and all other chemicals and reagents used were analytical grade.

Sample preparation

Caffeine at various concentrations prepared in normal distilled water, caffeic acid used to dissolve in 0.5% DMSO at various concentrations.

Drug design, drug likeness and bioavailability

Caffeine and caffeic acid smiles are retrieved from Pubchem database ID: CID 2519 and CID 689043. Molinspiration tool was used to draw combined (caffeine + caffeic acid) compound structure. The molecular properties of caffeine, caffeic acid and (caffeine + caffeic acid) were predicted using a software package (Molinspiration Cheminformatics, Slovensky Grob, Slovak Republic).

In vitro lipid peroxidation

Rat liver lipid peroxidation measured by Prasanth Kumar *et al.* (2008) Lipid peroxidation was initiated by adding 100 µl of 15 mM $FeSO_4$ solution to 3 ml of liver homogenate (final concentration was 0.5 mM). After 30 min. 100 µl of this reaction mixture was taken in a tube containing 1.5 ml of 10% TCA for 10 min. Then the tubes were centrifuged and supernatant was separated and mixed with 1.5 ml of 0.67% TBA in 50% acetic acid. The mixture was heated in a hot water bath at 85°C for 30 min and in a boiling water bath to complete the reaction. The intensity of pink coloured complex formed was measured at 532 nm in a spectrophotometer. The percentage inhibition of lipid peroxidation was calculated by following formula.

$$\text{Lipid peroxidation (\%)} = [(A_0\text{-}A_1)/A_0] \times 100$$

Where A_0 was the absorbance of the control reaction and A_1 was the absorbance in the presence of the sample of caffeine, caffeic acid and the combination.

DPPH radical scavenging activity

Free radical scavenging activity of the caffeine, caffeic acid and combination was measured by 1,1-diphenyl-2-picrylhydrazyl (DPPH) using the method described by Shimada *et al*. (1992). Briefly 0.1 mM solution of DPPH in methanol was prepared; 1 ml of the solution was added to 3 ml of the fraction in methanol at different concentrations (100-250 µg/ml). The mixture was shaken vigorously and allowed to stand and kept in dark at room temperature for 30 minutes. Then the absorbance was measured at 517 nm by using a UV-Visible Spectrophotometer. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The percent DPPH scavenging effect was calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = [(A_0\text{-}A_1)/A_0] \times 100$$

Where A_0 was the absorbance of the control reaction and A_1 was the absorbance in the presence of the sample of caffeine, caffeic acid and the combination.

Superoxide anion scavenging activity

Superoxide anion scavenging activity based on the method described by Liu *et al*. (1997) with slight modification. One ml of nitroblue tetrazolium (NBT) solution (156 mM/L NBT in 100 mM/L phosphate buffer, pH 7.4) 1ml NADH solution (468 mM/L in 100 mM/L phosphate buffer, pH 7.4) The reaction started by adding 100 ml of phenazine methosulphate (PMS) solution (60 mM/L PMS in 100 mM/L phosphate buffer, pH 7.4) to the mixture. The reaction mixture was incubated at 25ºC for 5 minutes, and the absorbance at 560 nm was measured against blank samples. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The percentage inhibition of superoxide anion generation was calculated using the following formula:

$$\text{Superoxide anion scavenging activity (\%)} = [(A_0\text{-}A_1)/A_0] \times 100$$

Where A_0 was the absorbance of the control reaction

and A1 was the absorbance in the presence of the sample of caffeine, caffeic acid and the combination.

Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activity according to Ruch. (1989) method Hydrogen peroxide solution (2 mM) was prepared with standard phosphate buffer (pH, 7.4). Compounds (100 ± 400 µg/ml) in distilled water were added to hydrogen peroxide solution (0.6 ml). Absorbance of hydrogen peroxide at 230 nm was determined after 10 minutes against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage scavenging of hydrogen peroxide of both the compounds and standard ascorbic acid compound was determined.

$$H_2O_2 \text{ scavenging effect (\%)} = [(A_0-A_1)/A_0] \times 100$$

Where A_0 was the absorbance of the control reaction and A_1 was the absorbance in the presence of the sample of caffeine, caffeic acid and the combination.

Reducing power ability

The reducing power of caffeine, caffeic acid and combination was determined by the method of Oyaizu (1986). Different concentrations of caffeic acid (100–250 µg/ml) in 1ml of distilled water were mixed with phosphate buffer (2.5 ml, 0.2 mol/l, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1%). The mixture was incubated at 50ºC for 20 minutes. Portions (2.5 ml) of trichloroacetic acid (10%) were added to the mixture. This then centrifuged for 10 minutes at 1000 g. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%), and the absorbance was measured at 700 nm in a spectrophotometer. Increased absorbance of the reaction mixture indicates an increase of reduction capability.

Determination of total antioxidant capacity

The total antioxidant capacity of the samples was evaluated by the phosphomolybdenum method according to the procedure of Prieto (1997). The assay is based on the reduction of Mo (VI) – Mo (V) by the compounds and subsequent formation of a green phosphate/Mo (V) complex at acid pH. 0.3 ml caffeine, caffeic acid and combined with 3ml of reagent solution (0.6M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95ºC for 90 minutes. Then the absorbance of the solution was measured at 695 nm using a spectrophotometer against blank after cooling to room temperature. The antioxidant activity is expressed as the number of equivalents of ascorbic acid.

Statistical analysis

All the experiments determined was expressed as the mean of three replicate and presented as mean ± SD (standard deviation). Statistical analysis was performed Duncan's Multiple Range Test (DMRT) as statistically significant levels were set at $p < 0.05$ using SPSS 16.0 software package.

Results and Discussion

Drug design, bioactivity score and drug-like properties

Caffeine, caffeic acid and (caffeine + caffeic acid) similes drawn and fixed the following is

Caffeine - cn1cnc2c1c (=o) n (c (=o) n2c) c

Caffeic acid - o=c(o)\c=c\c1cc(o)c(o)cc1

(Caffeine+Caffeicacid) - oc (=o) c=cc1=c (c (=c (c=c1) o) o) c [n] 2c=nc3=c2c (n (c (n3c) =o) c) =o

Caffeine and caffeic acid smiles were retrieved from Pubchem database. These compounds docked to each other and combined similes are obtained. Predicted by molinspiration is cheminformatic tool. Computational (In silico) methods widely using in pharmacology hypothesis development and testing These in silico methods include database searching, quantitative structure-activity relationships, similarity searching, computational modeling, optimization of novel molecules with affinity to a target, the clarification of absorption, distribution, metabolism, excretion and toxicity properties as well as physicochemical characterization according to Lipinski rules (2004).

Molinspiration-cheminformatics software tool

Structure-activity relationship, drug likeness properties of caffeine, caffeic acid and the combined structure has been analyzed by molinspiration tool. Synthetic and natural compounds using as drug against diseases need to under the rules and conditions, compounds were further evaluated for compliance with Lipinski's 'rule-of-five'. Lipinski (2004) according to this rules molecular pharmacokinetic properties of a compound that make it potentially applicable as an oral drug. Most drug-like molecules have log $P < 5$, molecular weight <500 and should contain not more than 10 hydrogen bond acceptors and less than 5 hydrogen bond donors. Molecules violating more than one of these rules may have problems with bioavailability (Lipinski *et al*., 1997) frequently the topological polar surface area (TPSA), the molecular volume,

lipophilicity and solubility, widely acknowledged as an important factor determining transport of drugs across membranes. Caffeine, caffeic acid and combination (caffeine + caffeic acid) having better drug-likeness properties and ADME properties and these compound pharmacokinetics properties not violating Lipinski rule. Insillico methods database searching, Structure-related activity, computational drug design and modeling. Such methods have seen frequent use in the discovery and optimization of novel molecules with affinity to a target, the clarification of absorption, distribution, metabolism, excretion and toxicity properties as well as physicochemical characterization (Ekins *et al*., 2007).

Invitro lipid peroxidation assay

Unsaturated lipids in liver tissue are very susceptible to peroxidation when they are exposed to ROS. In the present investigaion, we have incubated the liver tissue in presence of a ROS generating system, ascorbate/$FeSO_4$, and examned the effect on tissue homogenate by measuring the optical density (OD) at 532 nm. Reactive oxygen species can initiate lipid peroxidation and DNA damage leading to mutagenesis, carcinogenesis and cell death if the antioxidant potential is insufficient Thiobarbituric acid reactive substances (TBARS) assay which was originally developed for testing rancidity due to oxidized lipids is now widely used as an index for measuring lipid peroxidation. Figure 1 shows that the invitro lipid peroxidation activity exhibited the following order: ascorbic acid > caffeine> caffeic acid > caffeine + caffeic acid at the dose of maximum 400 µg/ml. Compared with the compounds caffeine shows that closely related result with standard ascorbic acid.

DPPH free radical scavenging activity

This is the most widely reported method for screening of *invitro* antioxidant activity of many drug compounds. DPPH assay method is based on the reduction of methanolic solution of colored free radical DPPH by free radical scavenger (Sanchez-Moreno *et al.*, 1999). In the DPPH assay, the antioxidants were able to reduce the stable radical DPPH to the yellow colored diphenyl-picrylhydrazine. The method is based on the reduction of alcoholic DPPH solution in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical form DPPH-H by the reaction. With this method it was possible to determine the anti radical power of an antioxidant by measuring of a decrease in the absorbance of DPPH at 517 nm (Matth¨aus *et al*., 2002). Ascorbic acid is the reagent used as standard. These compounds

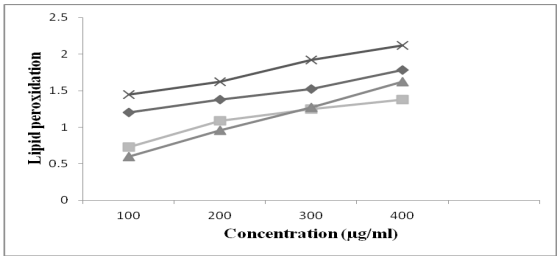

Figure 1. *Invitro* lipid peroxidation (rat liver) activity of caffeine, caffeic acid and combination at different concentrations (100-400 µg/ml), caffeine, caffeic acid, (caffeine + caffeic acid), ascorbic acid.

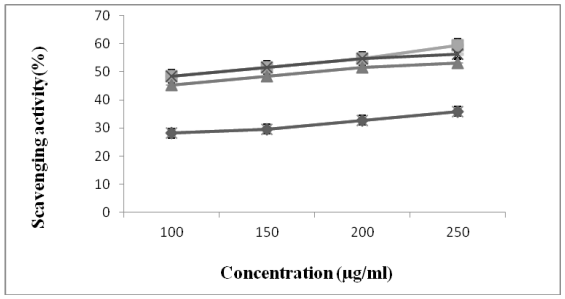

Figure 2. DPPHfree radical Scavenging activity of caffeine, caffeic acid and combination against 1,1-diphenyl-2 picrylhydrazyl radical at different concentrations (100-250µg/ml), caffeine, caffeic acid, (caffeine + caffeic acid), ascorbic acid.

are able to reduce the stable radical DPPH to the yellow colored diphenylpicrylhydrazine. Figure 2 shows that the free DPPH radical scavenging effect of these compounds and standard asorbic acid is in the following order: antioxidant properties of caffeic acid (48.37%) > caffeine + caffeic acid > (45.48%) > ascorbic acid (40%) > caffeine (28.22%) at the dose of maximum 250 µg/ml. DPPH free radical activity also increased with increasing concentration in a dose dependant manner and caffeic acid higher activity than others. We observed from the results caffeine, caffeic acid and combination and the relatively stable DPPH radical has been widely used to test the ability of compounds to act as free radical-scavengers or hydrogen donors.

Superoxide anion scavenging activity

Superoxide anion has been implicated in several pathophysiological processes due to its transformation into more reactive species such as hydroxyl radical that initiate lipid peroxidation (Wickens *et al.*, 2001). Superoxide anion plays an important role in the formation of other ROS such as hydrogen peroxide, hydroxyl radical, and singlet oxygen, which induce oxidative damage in lipids, proteins and DNA (Pietta *et al.*, 2000). Superoxide anion derived from dissolved oxygen by PMS–NADH coupling

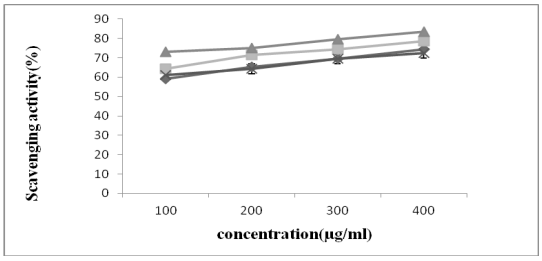

Figure 3. Super oxide scavenging activities of caffeine, caffeic acid and combination at different concentrations (100-400µg/ml), caffeine, caffeic acid, (caffeine + caffeic acid), ascorbic acid.

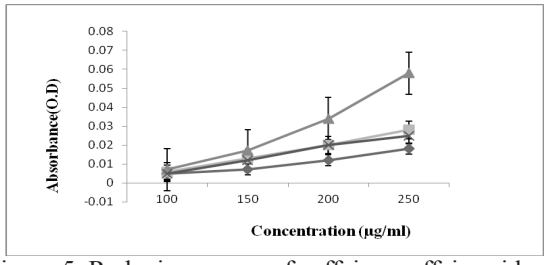

Figure 5. Reducing power of caffeine, caffeic acid and combination at different concentration (100-250µg/ml), caffeine, caffeic acid, (caffeine + caffeic acid), ascorbic acid.

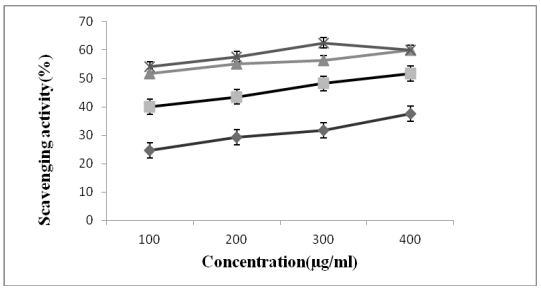

Figure 4. Hydrogen peroxide scavenging activities of caffeine, caffeic acid and combination at different concentration (100-400 µg/ml), caffeine, caffeic acid, (caffeine + caffeic acid), ascorbic acid.

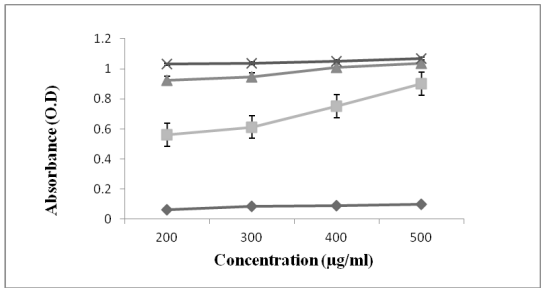

Figure 6. Total antioxidant of caffeine, caffeic acid and combination different concentration (200-500µg/ml), caffeine, caffeic acid, (caffeine + caffeic acid), ascorbic acid.

reaction reduces NBT in this system. In this method, superoxide anion reduces the yellow dye (NBT^{2+}) to produce the blue formazan which is measured spectrophotometrically at 560 nm and antioxidants are able to inhibit the blue NBT formation (Parejo *et al*., 2002). The decrease of absorbance at 560 nm with antioxidants indicates the consumption of superoxide anion in the reaction mixture. Figure 3 shows that the superoxide anion scavenging activity properties of compounds and standard ascorbic acid is in the following order: caffeine + caffeic acid (83.36%) > caffeic acid (78.57%) > caffeine (74.48%) > ascorbic acid (72.44%) at the dose of maximum 400 µg/ml. our results, All the three compounds have strong superoxide radical activity than ascorbic acid and the combination (caffeine + caffeic acid) has exhibits higher superoxide radical scavenging activity than caffeine, caffeic acid and ascorbic acid.

Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activity of a compound may serve as a significant indicator of its potential antioxidant activity. In the body, H_2O_2 is rapidly decomposed into oxygen and water and this may produce hydroxyl radicals ($OH^{\cdot}$) Hydrogen peroxide has the ability to penetrate biological membranes (Wu *et al.*, 1996), Hydrogen peroxide is a weak oxidizing agent that inactivates a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. It can cross cell membranes rapidly and inside the cell, it can probably react with Fe^{2+} and possibly Cu^{2+} ions to form hydroxyl radicals ($OH^{\cdot}$) that can initiate lipid peroxidation and cause DNA damage and it may be the origin of many of its toxic effects (Miller *et al.*, 1993). Compounds concentration (100 ± 400 µg/ml) in distilled water is added to hydrogen peroxide and absorbance at 230 nm is determined after 10 minutes against a blank solution containing phosphate buffer without hydrogen peroxide. Hydrogen peroxide scavenging activity of the compounds increased with increase the concentration in dose dependant manner. The ability of caffeine, caffeic acid and caffeine + caffeic acid to hydrogen peroxide scavenges when compared with ascorbic acid was significantly decreased. Figure 4 shows that the hydrogen peroxide scavenging activity properties of these compounds and standard asorbic acid is in the following order: ascorbic acid (64.7%) > caffeine + caffeic acid (60%) > caffeic acid (51.7%) > caffeine (37.64%) at the dose of maximum 45 0µg/ml.

Reducing power activity

Reducing the capacity of a compound may serve as a significant indicator of its potent antioxidant activity. Yellow colour of the test solution changes to various shades of green and blue depending upon the reducing power of samples. The presence of antioxidant substances in the compound samples causes the reduction of the Fe^{3+} ferricyanide complex

to the ferrous form. Therefore, Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm (Chung *et al*., 2002). The reducing properties are generally associated with the presence of reductones (Duh *et al.*, 1998), which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom and the activities of antioxidants have been attributed to various mechanisms such as prevention of chain initiation, decomposition of peroxides, reducing capacity and radical scavenging (Yildirim *et al.*, 2000). The results show that reducing power of caffeine, caffeic acid and combination were increased with increase concentration. Caffeine + caffeic acid shows an effective reducing power than caffeine and caffeic acid. Figure 5 shows reducing power capability of compounds and standard ascorbic acid is in the following order: caffeine + caffeic acid > caffeic acid > ascorbic acid > caffeine at the dose of maximum 250µg/ml.

Total antioxidant activity

The phosphomolybdate method has been routinely used to evaluate the total antioxidant capacity of the extracts and compounds (Prieto *et al*., 1997). The phosphomolybdenum method is quantitative since the antioxidant activity is expressed as the number of equivalents of ascorbic acid and the phosphomolybdenum method was based on the reduction of Mo (VI) to Mo (V) by the antioxidant compound and the formation of a green phosphate/ Mo (V) complex with a maximal absorption at 695 nm. Total antioxidant capacity of caffeine, caffeic acid and caffeine + caffeic acid is expressed as the number of equivalents of ascorbic acid. The results indicate a concentration dependent total antioxidant capacity of the caffeine, caffeic acid and caffeine + caffeic acid is in the increased with the increasing concentration of the compounds. Figure 6 shows that the total antioxidant of activity exhibited the following order: ascorbic acid > caffeine + caffeic acid > caffeic acid > caffeine at the dose of maximum 500 µg/ml.

Conclusion

The results of lipid peroxidation and free radical scavenging evaluation based different assay showed that caffeine, caffeic acid and combination (caffeine, caffeic acid) effectively involved in free radical scavenging activity, which may be helpful in preventing or slowing progress of various oxidative stress related diseases. Such a dietary compound could be a potential source of natural antioxidant that could have great importance as therapeutic agent. The finding of lipid peroxidation and free radical-scavenging activity of caffeine, caffeic acid and their combination prompted us to test future studies.

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