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Advances in Pharmacology and Clinical Trials
ISSN: 2474-9214

Comparative Analysis of Free Radical Scavenging Potential of Pyrroloquinoline Quinone (PQQ) and Several Plants Extracts by *in-vitro* Methods

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Research Article

Volume 2 Issue 1

Received Date: June 21, 2017

Published Date: July 11, 2017

Abstract

In-vitro lipid peroxidation (LPO) was induced by ferrous sulphate ($FeSO_4$), hydrogen peroxide (H_2O_2) and carbon tetrachloride (CCl_4) and then the effects of five different concentrations (10, 20, 40, 80 and 160 µM) of pyrroloquinoline quinone (PQQ) were evaluated in liver, the major target organ of a drug. A comparison was made with the effects of some known antioxidative plant extracts and vitamin C. For this different concentrations of PQQ, vitamin C and herbal extracts of *Annona squamosa* (AS), *Rauvolfia serpentina* (RS), *Withania somnifera* (WS), *Commiphora mukul* (CM), *Syzygium cumini* (SC) and *Gymnema sylvestre* (GS) were considered and examined through different *in-vitro* antioxidant potential assays such as Azino-bisethylbenzothiazoline-6-sulphonic acid (ABTS) scavenging assay; Diphenylpicrylhydrazyl (DPPH) scavenging assay; Metal ($FeCl_2$) chelating assay; Hydrogen peroxide (H_2O_2) scavenging assay and Superoxide (SO) radical scavenging assay. While $FeSO_4$, H_2O_2 and CCl_4 markedly enhanced the hepatic LPO; simultaneous administration of PQQ reduced it in a concentration dependent manner. This effect was observed in all three, $FeSO_4$, H_2O_2 and CCl_4 induced hepatic LPO. Out of five different concentrations of PQQ, 20 µM and 80 µM showed the maximum inhibition in LPO, suggesting its beneficial/antioxidative activity. While comparing the antioxidative potential of PQQ with some known antioxidative herbal extracts and vitamin C, the test drug exhibited highest antioxidative activity in all the above free radical scavenging assays, further consolidating very high antioxidative potential of PQQ. PQQ exhibited better antioxidative potential than some known plant extracts. Therefore, its therapeutic use may prove to be advantageous in ameliorating oxidative stress associated diseases.

Keywords: PQQ; TBARS; ABTS; DPPH; SO; H_2O_2; Metal chelating assay

Introduction

It is now well known that the lipid peroxidation (LPO) is induced by free radicals and reactive oxygen species that are generated continuously in the physiological processes of all living systems [1] and if not scavenged or converted to less reactive forms, they attack the unsaturated bond of the macromolecules, ultimately damaging the cell [2]. It is a growing belief that most of the common health problems are associated with

enhanced LPO [3-7]. Few investigations are there on the antiperoxidative effects of some plant extracts involving both *in vivo* and *in vitro* studies [8-11]. Although pyrroloquinoline quinone (PQQ) is believed to be an antioxidant, it has not been affirmed. In order to consolidate its antioxidative potential, in the present investigation, for the first time an attempt has been made to study the hitherto unknown *in vitro* antiperoxidative effects of PQQ, using some antioxidative agents.

It is well established that iron is involved in lipid peroxidation. As ferrous ions precipitate the formation of oxygen radicals and initiate peroxidative process, ferrous sulphate ($FeSO_4$) is often used to induce tissue LPO [12]. Similarly, hydrogen peroxide (H_2O_2) and carbon tetrachloride (CCl_4) have also been used to induce tissue LPO [13,14]. Therefore, in this investigation the LPO was induced by $FeSO_4$, H_2O_2 and CCl_4 the efficacy tests were made considering inhibition of LPO in hepatic tissues.

The antiperoxidative effects of some plant extracts were also correlated with free radical scavenging activity of PQQ. Phytochemical investigations have shown the presence of alkaloids, saponins, D-mannitol, betulic acid and β-sitosterol in some plants [15]. Because phytochemicals such as polyphenols, flavonoids, anthraquinones are known to exhibit antioxidative properties [16-18], plant extracts are believed to act as antioxidant. PQQ is also considered as antioxidant, but its antioxidant potential has not been consolidated. In-fact, its *in vitro* antioxidative potential is still unclear. In the present study we have investigated their antioxidative potential *in vitro* and compared the same with some known plant extracts, considering vitamin C (Vit.C) as a standard.

Materials and methods

Animal

Standard ethical guidelines of the committee for the purpose of control and supervision of experiments on animals (CPCSEA), Ministry of Environment, Forest and Climate Change, New Delhi, Govt. of India. (Reg. No. 779/Po/Ere/S/03/CPCSEA) were followed. Before starting the investigation, the approval of the departmental ethical committee for handling and maintenance for experimental animals was also obtained.

Chemicals

Azino-bisethylbenzothiazoline-6-sulphonic acid (ABTS); Diphenylpicrylhydrazyl (DPPH) was obtained from SIGMA, USA. Thio-barbeuteric acid (TBA) was procured from Hi-media pvt. Ltd. $FeCl_2$, $FeSO_4$, H_2O_2, CCl_4 and all other chemicals (analytical grades) were obtained from Merck India Ltd., Mumbai, India. While $FeSO_4$ was dissolved in distilled water, H_2O_2 and CCl_4 were dissolved in phosphate buffer saline and dimethyl sulfoxide (DMSO), respectively, as used earlier [11,19].

Preparation of the herbal extracts

Officinal parts of six different herbs (Table 1) which were reported to act as antioxidant [7,9] were collected from the local market (Indore, India),dried then pulverized in an electrical grinder to obtain a free flowing dry powder.

(Common name)			
Scientific name	**English**	**Hindi**	**Family**
Annona squamosa	(Sugar apple)	(Sitafal)	Annonaceae
Rauvolfia serpentina	(Snake root)	(Sarpagandha)	Apocynaceae
Withania somnifera	(Winter cherry)	(Ashwagandha)	Solanaceae
Commiphora mukul	(Gum gugul)	(Guggul)	Burseraceae
Syzygium cumini	(Black plum)	(Jambul)	Myrtaceae
Gymnema sylvestre	(Australian cowplant)	(Gurmar)	Apocynaceae

Table 1: Botanical species were used for the comparative study.

100 grams of powder of each plant part was extracted with 400 ml of ethyl alcohol (70%) at the RT, incubated overnight and then filtered through Whatman filter paper no.1. The dried filtrate (at 37 ºC) was stored for future use. Each extract powder was dissolved in double distilled water (DW) for final experimentation.

Preparation of Liver Homogenate

For this adult male rat were sacrificed after anaesthetizing with mild chloroform. Liver from each animal was taken out immediately, blood clots were removed, washed in phosphate buffered saline (PBS), cut into small pieces and then homogenized in 10% ice cold PBS. Different experiments were performed with the prepared liver homogenates and at the end LPO was measured using the standardized protocol followed in our laboratory [9,11].

Induction of LPO

LPO was studied using the standardized protocol routinely followed in our laboratory [9,11]. In brief, to 1 ml of liver homogenate, 100 µl $FeSO_4$ /H_2O_2 respectively and CCl_4 (20 µl for CCl_4 case) were added; while in control set, the same amount of DW was mixed. Then the reaction mixture was incubated at 37°C for 1 h, following which 2 ml TCA (10%) was added to the mixture and the samples were centrifuged at 3000 rpm for 5 minutes. 2 ml supernatant was taken out and to it 1 ml TBA was added followed by boiling in water bath for 45 min. After cooling in running water OD was taken at 532 nm as routinely done in our laboratory [7,11].

LPO in $FeSO_4$ System

In three sets of test tubes (in triplicate), each containing 1 ml of liver homogenate, three different concentrations (1 or 5 or 10 mM) of $FeSO_4$ were taken. These concentrations of $FeSO_4$ were taken from earlier report [11]. A control set was also run in which all materials other than $FeSO_4$ were added. All the tubes were processed for the estimation of LPO by TBA reaction method as described above. Considering the effective concentration of $FeSO_4$ that showed maximum increase in hepatic LPO, antiperoxidative effect of the different concentrations of PQQ was evaluated. Five concentrations of PQQ were considered in this experiment that was 10, 20, 40, 80 and 160 µM. All five concentrations were taken in triplicate. Simultaneously a set of drug control tubes was processed that contained all the materials except PQQ.

LPO was induced by addition of 100 µl of 5 mM $FeSO_4$ in the reaction mixture containing PBS and chopped tissue and by incubating at 37°C for 2 hour [7]. In another set liver slices were incubated with 100 µl of 5 mM $FeSO_4$ along with one of the concentrations (10, 20, 40, 80 and 160 µM) of PQQ dissolved in DW. After 2 h, each homogenate mixture of chopped liver was centrifuged at 800g and the supernatant was used to measure LPO by TBA reaction method, as followed earlier [11,20]. A control set was run in which all materials other than $FeSO_4$ or PQQ were added.

LPO in H_2O_2 and CCl_4 System

Similar procedure, as mentioned above was repeated with H_2O_2 or CCl_4. In three sets of test tubes (each in triplicate) containing 1 ml of liver homogenate, three different concentrations (40, 80 and 160 mM) of H_2O_2 or three different concentrations (10, 20 and 40 µl) of CCl_4 were taken. These concentrations of H_2O_2 and CCl_4 were taken from earlier report [7,11]. A control set was also run in which all materials other than H_2O_2 or CCl_4 were added. All the tubes were processed for the estimation of LPO by TBA reaction method as described above.

Considering the most effective concentration of H_2O_2 or CCl_4 that showed maximum increase in hepatic LPO, antiperoxidative effects of PQQ was evaluated. Five concentrations of PQQ were considered in this experiment that was 10, 20, 40, 80 and 160 µM. All five concentrations were taken in triplicate; a set of drug control tubes was also processed that contained all the materials except PQQ. LPO was estimated with similar protocol as mentioned earlier. In the H_2O_2 system, the reaction mixture contained 400 mg of the chopped liver tissues in 3.9 ml of PBS, 100 µl of 80 mM H_2O_2 and PQQ of one of the five concentrations, 10, 20, 40, 80 and 160 µM (each in triplicates). The mixture was incubated at 37 ºC for 2 h. Following the addition of trichloroacetic acid (TCA) and TBA the optical density (OD) was measured at 532 nm [20]. In case of CCl_4 system also the reaction mixture contained 400 mg of the chopped liver tissues in 3.9 ml of PBS, 20 µl CCl_4 (1:4 in DMSO, v/v) and PQQ of one of the five concentrations, 10, 20, 40, 80 and 160 µM (each in triplicates). Following the incubation at 37 ºC and the addition of TCA and TBA, OD was measured at 532 nm [21].

Antioxidant Activity Determinations

Azino-bisethylbenzothiazoline-6-sulphonic acid (ABTS) Scavenging Assay:

For ABTS assay, the procedure followed was of Arnao et al. [22], with some modifications. The stock solutions included 7.4 mM ABTS solution and 2.6 mM potassium per sulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1mL ABTS solution with 60 ml. Then the absorbance was taken at 734 nm using the

spectrophotometer. The assay was performed at least in triplicate. Controls containing 990 µl of PBS, to replace ABTS, were used to measure absorbance of the extract themselves. The assay relies on the antioxidant capability of the samples to inhibit the oxidation of ABTS to ABTS•+ radical cat-ion. The percentage of ABTS scavenging was calculated as:

% ABTS scavenging = [(AC – AS)/AC] x 100

Where, AC is the absorbance of the control and AS is the absorbance of the sample.

Diphenylpicrylhydrazyl (DPPH) scavenging assay: The effect of crude extracts on the DPPH radicals was estimated using the method of Yamaguchi et al., [23]. An aliquot of crude extract (30 µl) and glutathione (GSH) (0.5 mg/ml, 30 µl) were mixed with 100 mM Tris-HCl buffer (120 µl, pH 7.4) and then with 150 µl of the DPPH in ethanol to a final concentration of 250 µM. The mixture was shaken vigorously and left to stand at room temperature for 20 min in the dark. The absorbance at 517 nm of the reaction solution was measured spectrophometrically. The percentage of DPPH decolourization of the sample was calculated according to the following equation:

% decolourization = [(AC – AS)/AC] x 100

Where, AC is the absorbance of the control and AS is the absorbance of the sample.

Metal chelating activity: The chelating of ferrous ions by extracts was estimated by using the method of Ebrahimzadeh et al., [24]. For iron chelating activity assay, the reaction mixture containing 1 ml O-Phenanthroline, 2 ml ferric chloride and 2 ml of test extract at various concentrations ranging from 2 to 1000 µg/ml in a final volume of 5 ml was incubated for 10 minutes at ambient temperature. The absorbance at 510 nm was recorded. Ascorbic acid was added instead of extract and absorbance obtained was taken as equivalent to 100% reduction of all ferric ions. Blank was carried out without drug. Metal chelating activity was calculated following the formula:

% Metal chelating activity = [(AC – AS)/AC] x 100

Where, AC is the absorbance of the control and AS is the absorbance of the sample.

Hydrogen peroxide (H_2O_2) scavenging assay: Hydrogen peroxide scavenging activity was determined according to the method of Ruch et al. [25]. A solution of H_2O_2 (40 mM) was prepared in phosphate buffer (pH 7.4). Test extract (100 µg/ml) in distilled water was added to a H_2O_2 solution (0.6 mL, 40mM) and absorbance at 230 nm was determined 10 minutes later against a blank solution containing the phosphate buffer without H_2O_2. The percentage of H_2O_2 scavenging was calculated as:

% Scavenged [H_2O_2] = [(AC – AS)/AC] x 100

Where AC is the absorbance of the control and AS is the absorbance of the sample.

Superoxide (SO) radical scavenging assay: The superoxide radical scavenging activity was studied by using the method of Liu, et al. [26]. 100 µl Riboflavin solution (20 µg), 200 µl EDTA solution (12 mM), 200 µl methanol and 100 µl NBT (Nitro-blue tetrazolium) solution (0.1 mg) were mixed in test tube and reaction mixture was diluted up to 3 ml with phosphate buffer (50 mM). The absorbance of solution was measured at 590 nm in a spectrophotometer (Shimadzu, UV-1800, Japan). First time, the percentage scavenging activity was calculated using the following formula,

% Scavenged [SO] = [(AC – AS)/AC] x 100

Where, AS is the absorbance of the test (With extract) and AC is the absorbance of the control (without extract).

Statistical Analysis

Data are expressed as mean ± SE. Statistical analysis was done by using analysis of variance (ANOVA) followed by student's t-test and P values of 5% and less were considered to be significant.

Results

Following the incubation of liver homogenates with 1, 5 and 10 mM of $FeSO_4$ a significant increase in LPO ($P<0.001$ to all) was observed. However, the maximum % of LPO increased was observed at 5 mM of $FeSO_4$ (i.e. 394%). When considering this effective concentration of $FeSO_4$ that showed maximum increase in hepatic LPO (i.e 5mM), antiperoxidative effect of the test compound (PQQ) was evaluated. $FeSO_4$ (5 mM) markedly enhanced hepatic LPO ($P< 0.001$), following the incubation with five different concentrations of PQQ, (i.e 10, 20, 40, 80 and 160 µM) could inhibit the $FeSO_4$ (5 mM) induced LPO in all. However, the maximum decrease was observed at 20 µM ($P< 0.001$, Figure 1; as compared to the average value of $FeSO_4$ control tubes). The percentage decrease in LPO of different concentrations of PQQ (10, 20, 40, 80, and 160 µM) were 50%, 67%, 58%, 60% and 60% respectively (Figure 1).

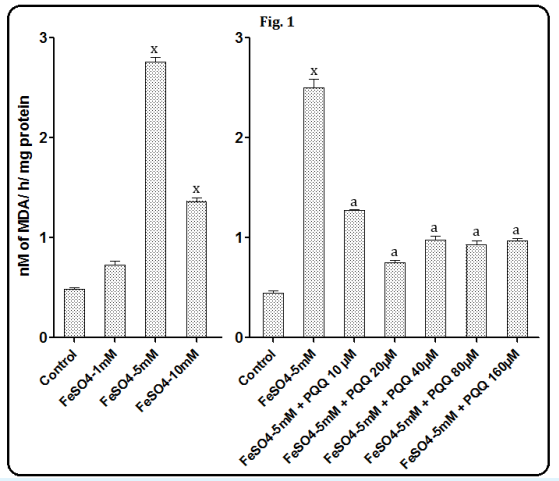

Figure 1: Effects of PQQ at 10, 20, 40, 80 and 160 µM on FeSO4-induced hepatic LPO. Data are mean ± SEM (n=3). x, P< 0.001 compared to the respective control values. a, P< 0.001 as compared to the respective FeSO4 treated value.

Incubation of rat liver slices with different concentrations of H_2O_2 (40, 80 and 160 mM) resulted in a significant increase in hepatic LPO. However, maximum the percent increase i.e. 363% was observed at 80 mM. Considering this effective concentration of H_2O_2 to induce hepatic LPO, i.e 80 mM, when incubation with any of the five different concentrations of PQQ, i.e 10, 20, 40, 80 and 160 µM was done, the maximum decrease was observed at 80 µM (P< 0.001, as compared to the average value of H_2O_2 control tubes. The percent decreases in LPO of different concentrations of PQQ (10, 20, 40, 80, 160 µM) were 3%, 2%, 24%, 62% and 57% respectively (Figure 2).

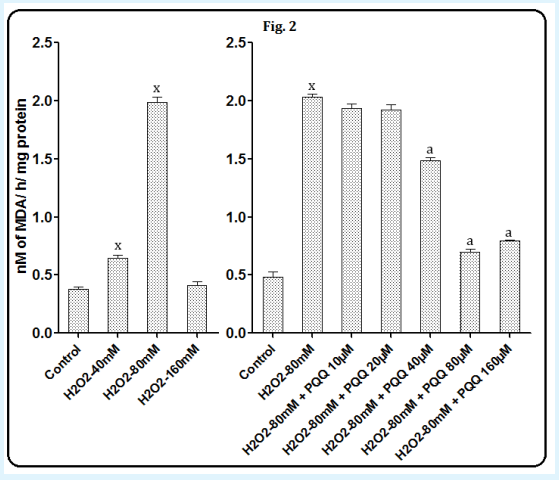

Figure 2: Effects of PQQ at 10, 20, 40, 80 and 160 µM on H_2O_2-induced hepatic LPO. Data are mean ± SEM (n=3). x, P< 0.001 compared to the respective control values. a, P< 0.001 as compared to the respective H_2O_2 treated value.

With respect to incubation of liver homogenates with CCl_4 (10, 20 and 40 µl), a significant increase in LPO (P < 0.001 to all) was observed in all three concentrations (40%, 407% and 175% respectively). When, considering the effective concentration of CCl_4 that showed maximum increase in hepatic LPO (i.e 20 µl), antiperoxidative effects of the test compound (PQQ) was evaluated, CCl_4 (20 µl) markedly enhanced hepatic LPO (P<0.001). However, following the incubation with five different concentrations of PQQ, i.e 10, 20, 40, 80 and 160 µM, the maximum decrease was observed at 80 µM (P<0.001, as compared to the average value of CCl_4 control tubes). The other concentrations were also able to inhibit LPO but slightly less as compared to that of 80 µM. The percentage decreases in LPO of different concentrations of PQQ (10, 20, 40, 80, 160 µM) were 25%, 36%, 38%, 62% and 61% respectively (Figure 3).

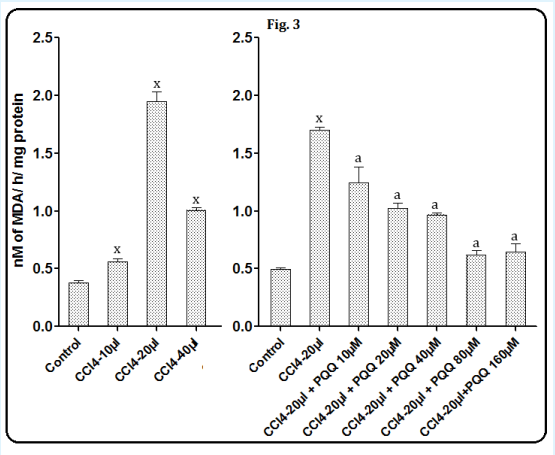

Figure 3: Effects of PQQ at 10, 20, 40, 80 and 160 µM on CCl_4-induced hepatic LPO. Data are mean ± SEM (n=3). x, P< 0.001 compared to the respective control values. a, P< 0.001 as compared to the respective CCl_4 treated value.

Thus, concentration dependent effects were observed with PQQ that inhibited $FeSO_4$/ CCl_4/ H_2O_2-induced hepatic LPO. After incubating liver homogenates with pre-standardized concentrations of $FeSO_4$ (5 mM), CCl_4 (20 µl) and H_2O_2 (80 mM); there was an increase in LPO. However, out of five different concentrations of PQQ, only 20 µM, 80 µM and 80 µM were found to inhibit maximally the $FeSO_4$/ CCl_4/ H_2O_2-induced tissue LPO respectively.

With respect to different antioxidant assays using PQQ, plant extracts and vitamin C; the results indicated that all six herbal extracts were effective in the radical scavenging

assays. However, differential effects were found in PQQ and the test plant extracts. Interestingly PQQ exhibited better effects as compared to the plant extracts and Vit C.

ABTS assay: The ABTS radical scavenging assay revealed that all three different concentrations of PQQ (25, 50, and 100 µg/ml) showed a marked scavenging of ABTS radicals (78.0%, 82.04% and 88.05%, respectively); which are significantly (P< 0.001 in all doses) higher as compared to that of plant extracts and Vit C. While RS & SC also showed the significant (P< 0.001) higher radical scavenging potential at 100 µg/ml (67.83 & 65.54% respectively) as compared to Vit C (Table 2).

Radical scavenging activity (%)			
PQQ & Plant extracts	**25 µg/ml**	**50 µg/ml**	**100 µg/ml**
WS	36.39 ± 0.34	44.19 ± 0.73	48.07 ± 0.33
CM	45.71 ± 0.15	52.72 ± 0.21	56.71 ± 0.36
RS	47.3 ± 0.09^{y}	51.3 ± 0.09	67.83 ± 0.42^{x}
AS	36.8 ± 0.18	44.73 ± 0.21	55.84 ± 0.25
SC	38.86 ± 0.25	54.42 ± 0.12	65.54 ± 0.08^{x}
GS	37.9 ± 0.11	46.55 ± 0.14	51.33 ± 0.08
PQQ	78.01 ± 0.08^{x}	82.04 ± 0.08^{x}	88.05 ± 0.33^{x}
Vit.C	45.97 ± 0.15	51.46 ± 1.51	58.12 ± 0.07

Table 2: Radical scavenging activity (%) in various plant extracts, Vit.C and PQQ observed by ABTS assay system.
^{WS}W. somnifera; ^{CM}C. mukul; ^{RS}R. serpentina; ^{AS}A. squamosa; ^{SC}S.cumini; ^{GS}G. Sylvestre, ^{PQQ} Pyrroloquinoline quinone ; Data are expressed in % inhibition (mean ± SE; n=3). ^{x} P<0.001; ^{y} P<0.01 and ^{z} P<0.05 significantly more effective as compared to the respective concentration of Vit.C.

DPPH assay: In DPPH scavenging assay, all the studied doses of PQQ (25, 50, and 100 µg/ml) were found to be most effective (with a percent scavenging activity of 81.76, 88.21 and 91.69 % respectively), PQQ significantly (P< 0.001 in all doses) exhibited better radical scavenging potential as compared to that of Vit.C. RS & AS were found to show significant (P< 0.001) higher radical scavenging potential at 100 µg/ml (81.54 & 82.74% respectively) as compared to Vit.C (Table 3).

Radical scavenging activity (%)			
PQQ & Plant extracts	**25 µg/ml**	**50 µg/ml**	**100 µg/ml**
WS	72.69 ± 0.03	77.41 ± 0.19	82.11 ± 0.04^{x}
CM	35.18 ± 0.55	51.23 ± 1.05	67.66 ± 0.58
RS	72.45 ± 0.28	82.91 ± 0.94^{z}	81.54 ± 0.17^{z}
AS	77.38 ± 0.13	79.21 ± 0.49	82.74 ± 0.03^{x}
SC	77.34 ± 0.40	77.07 ± 0.19	78.25 ± 0.08
GS	75.43 ± 0.06	77.81 ± 0.25	80.75 ± 0.21
PQQ	81.76 ± 0.01^{x}	88.21 ± 0.10^{x}	91.69 ± 0.36^{x}
Vit.C	76.52 ± 0.22	77.75 ± 0.23	80.47 ± 0.06

Table 3: Radical scavenging activity (%) in various plant extracts, Vit.C and PQQ observed by DPPH assay system.
^{WS}W. somnifera; ^{CM}C. mukul; ^{RS}R. serpentina; ^{AS}A. squamosa; ^{SC}S.cumini, ^{GS}G. Sylvestre, ^{PQQ}Pyrroloquinoline quinone ; Data are expressed in % inhibition (mean ± SE; n=3). ^{x} P<0.001; ^{y} P<0.01 and ^{z} P<0.05 significantly more effective as compared to the respective concentration of Vit.C.

Metal chelating assay: In metal chelating activity, again all different concentrations of PQQ (25, 50, and 100 µg/ml) showed a greater metal chelating activities (43.68, 48.66 and 61.12%, respectively), which are significantly (P< 0.05; P< 0.01; P< 0.001 respectively) higher as compared to plant extracts and Vit.C. Of course no plant extract was found to exhibit better chelating activity as compared to Vit.C (Table 4).

Radical scavenging activity			
PQQ & Plant extracts	**25 µg/ml**	**50 µg/ml**	**100 µg/ml**
WS	39.11 ± 0.01	40.26 ± 0.16	41.79 ± 0.05
CM	42.69 ± 0.11	42.19 ± 0.03	41.44 ± 0.10
RS	25.46 ± 0.17	32.86 ± 0.07	38.17 ± 0.11
AS	6.08 ± 0.21	8.35 ± 0.26	23.86 ± 0.82
SC	7.8 ± 0.07	16.4 ± 0.15	29.66 ± 0.12
GS	38.84 ± 0.10	35.68 ± 0.15	29.56 ± 0.12
PQQ	43.68 ± 0.19^{z}	48.66 ± 0.18^{y}	61.12 ± 0.11^{x}
Vit.C	42.38 ± 0.08	42.55 ± 0.07	41.94 ± 0.01

Table 4: Radical scavenging activity (%) in various plant extracts, Vit. C and PQQ observed by metal chelating activity assay system.

WS,W. sominefera; CM,C. mukul; RS,R. serpentina; AS,A. squamosa; SC,S.cumini, GS, G. Sylvestre, PQQ, Pyrroloquinoline quinone; Data are expressed in % inhibition (mean ± SE; n=3). x, P<0.001; y, P<0.01 and z, P<0.05 significantly more effective as compared to the respective concentration of Vit.C.

SO radical scavenging assay: In SO radical scavenging assay, PQQ in all tested doses (25, 50, and 100 µg/ml) was found to be effective to scavenge superoxide radicals significantly (P< 0.001 in all doses) as compared to Vit.C, with a percent scavenging activity of 60.65, 66.32 and 72.14 % respectively, while no plant extract was found to have better chelating activity as compared to Vit C (Table 5).

Radical scavenging activity			
PQQ & Plant extracts	**25 µg/ml**	**50 µg/ml**	**100 µg/ml**
WS	6.02 ± 0.03	12.52 ± 0.06	36.66 ± 0.04
CM	9.01 ± 0.02	17.66 ± 0.07	37.78 ± 0.57
RS	11.8 ± 0.12^{x}	35.73 ± 0.06^{x}	39.9 ± 0.02
AS	14.85 ± 0.04^{x}	32.74 ± 0.04	45 ± 0.04
SC	11.68 ± 0.07^{x}	39.28 ± 0.09^{x}	47.99 ± 0.03^{x}
GS	37.22 ± 0.57^{x}	47.56 ± 0.21^{x}	54.34 ± 0.03^{x}
PQQ	17.66 ± 0.07^{x}	39.84 ± 0.04^{x}	63.31 ± 0.05^{x}
Vit.C	10.1 ± 0.03	32.8 ± 0.05	44.98 ± 0.03

Table 5: Radical scavenging activity (%) in various plant extracts, Vit.C and PQQ observed by H_2O_2 scavenging assay system.

WS,W. sominefera; CM,C. mukul; RS,R. serpentina; AS,A. squamosa; SC,S.cumini, GS, G. Sylvestre, PQQ,Pyrroloquinoline quinone; Data are expressed in % inhibition (mean ± SE; n=3). x, P<0.001; y, P<0.01 and z, P<0.05 significantly more effective as compared to the respective concentration of Vit C.

H_2O_2 scavenging assay: In this assay, GS was found to be most effective in H_2O_2 scavenging activity at dose of 25 and 50 µg/ml, as compared to PQQ and Vit C. However, at dose of 100 µg/ml PQQ was again found to be most effective (P< 0.001) to scavenge free radicals as compare to plant extracts and Vit C. While no plant extract was found to exhibit better chelating activity as compared to Vit C (Tables 6 and 7).

Radical scavenging activity (%)			
PQQ & Plant extracts	**25 µg/ml**	**50 µg/ml**	**100 µg/ml**
WS	23.99 ± 0.21	31.24 ± 0.02	38.51 ± 0.02
CM	28.41 ± 0.09	34.09 ± 0.03	42.98 ± 0.02
RS	38.36 ± 0.11	46.68 ± 0.15	55.33 ± 0.02

AS	32.66 ± 0.15	38.45 ± 0.03	48.01 ± 0.02
SC	39.53 ± 0.12	44.09± 0.02	50.36 ± 0.03
GS	43.76 ± 0.20	51.26 ± 0.04	57.01 ± 0.02
PQQ	60.65 ± 0.15[x]	66.32 ± 0.06[x]	72.14 ± 0.02[x]
Vit.C	54.54 ± 0.13	62.35 ± 0.10	65.95 ± 0.02

Table 6: Radical scavenging activity (%) in various plant extracts, Vit.C and PQQ observed with by SO scavenging assay system.
[WS]W. somnifera; [CM]C. mukul; [RS]R. serpentina; [AS]A. squamosa; [SC]S.cumini, [GS] G. Sylvestre, [PQQ]Pyrroloquinoline quinone; Data are expressed in % inhibition (mean ± SE; n=3). [x] P<0.001; [y] P<0.01 and [z] P<0.05 significantly more effective as compared to the respective concentration of Vit.C.

	ABTS	**DPPH**	**$FeCl_2$**	**SO**	**H_2O_2**
WS	39.79	38.76	38.25	42.03	54.14
CM	39.38	43.5	37.14	42.02	53.33
RS	42.27	37.92	40.09	41.13	43.12
AS	41.92	38.32	52.97	41.71	45.37
SC	41.97	37.72	48.89	40.16	44.63
GS	40.06	38.25	33.96	39.97	40.78
PQQ	38.89	38.51	41.62	39.3	47.4
Vit.C	40	38.13	37.29	39.07	46.18

Table 7: IC50 (µg/ml) values of various plant extracts, Vit.C and PQQ in ABTS; DPPH; metal chelating, superoxide and hydrogen peroxide scavenging assay.
[WS]W. somnifera; [CM]C. mukul; [RS]R. serpentina; [AS]A. squamosa; [SC]S.cumini, [GS] G. Sylvestre, [PQQ]Pyrroloquinoline quinone; Data are expressed in % inhibition (mean ± SE; n=3).

Thus the differential effects were found in PQQ and the test plant extracts. Interestingly PQQ exhibited better effects as compared to the plant extracts and the orders of the effects exerted by different plant extracts in different assay systems and in different concentrations were as follows:

- **ABTS scavenging assay:**
  25 µg/ml - PQQ > RS > Vit.C > CM > SC > GS > AS > WS
  50 µg/ml - PQQ > SC > CM > Vit.C > RS > GS > AS > WS
  100 µg/ml- PQQ > RS > SC > Vit.C > CM > AS > GS > WS
- **DPPH scavenging assay**:
  25 µg/ml - PQQ > SC >AS > Vit.C > GS > WS > RS > CM
  50 µg/ml - PQQ > RS >AS > GS > Vit.C > SC > WS > CM
  100 µg/ml- PQQ > AS >WS > RS > GS > Vit.C > SC > CM
- **Metal chelating activity:**
  25 µg/ml - PQQ > CM > Vit.C > WS > GS > RS > SC > AS
  50 µg/ml - PQQ > Vit.C > CM > WS > GS > RS > SC > AS
  100 µg/ml - PQQ > Vit.C > WS > CM > RS > SC > GS > AS
- **Superoxide radical scavenging assay**:
  25 µg/ml - PQQ > Vit.C > GS > AS > SC > RS > CM > WS
  50 µg/ml - PQQ > Vit.C > GS > RS > SC > AS > CM > WS
  100 µg/ml- PQQ > Vit.C > GS > RS > SC > AS > CM > WS
- **H_2O_2 scavenging assay:**
  25 µg/ml - GS > PQQ > AS > RS > SC > Vit.C > CM > WS
  50 µg/ml - GS > PQQ > SC > RS > Vit.C > AS > CM > WS
  100 µg/ml- PQQ > GS > SC > Vit.C > AS > RS > CM > WS

Discussion

From the results it was revealed that hepatic lipid peroxidation was inhibited by the test compound, PQQ at one /or the other doses, indicating its antiperoxidative nature. However, the percent inhibition was dependent on the type of chemical oxidant, used for the induction of peroxidation process.

Following the addition of different types of LPO inducing chemicals, there was a marked induction in the LPO. Interestingly, when the oxidant was incubated with PQQ, concentration dependent effects were observed, as PQQ inhibited $FeSO_4$/ CCl_4/ H_2O_2-induced hepatic LPO. In-fact, after incubating liver homogenates with pre-standardized concentrations of $FeSO_4$ (5 mM), CCl_4 (20 µl) and H_2O_2 (80 mM) along with PQQ, LPO was decreased in all the tubes. Although, out of five different concentrations of PQQ, only 20 µM, 80 µM and 80 µM were found to inhibit maximally the $FeSO_4$/ CCl_4/ H_2O_2-induced tissue LPO respectively, PQQ exhibited its antioxidative effects all the time.

As $FeSO_4$-induced LPO is known to take place through ferryl perferryl complex [28] and PQQ inhibited the $FeSO_4$-induced LPO in a dose dependent manner, it appears that the process was mediated through an inhibition of ferryl per-ferryl complex formation. It is also possibile that the total amount of ferrous ions available for LPO stimulation might have been partly reduced by PQQ to the forms that do not stimulate LPO.

The addition of H_2O_2 and CCl_4 also increased LPO significantly as observed earlier by other workers [9,11,28-30]. Interestingly, in these cases also the chemical-induced LPO was inhibited by PQQ again supporting its antiperoxidative nature. H_2O_2, a nonradical reactive oxygen species, considered as the most stable intermediate easily passes through cell membranes by diffusion and inside the cell it reacts with transition metals liberating hydroxyl radicals [31], which in turn, induce peroxidation of lipids and proteins, affecting cell integrity [31,32]. Probably in the present study an inhibition in H_2O_2-induced LPO by PQQ might have been mediated through the inhibition in OH radicals.

CCl_4-induced hepatic LPO was also inhibited by PQQ, further supporting its antiperoxidative potential. CCl_4 is believed to be metabolized by cytochrome P_{450} present in the microsomal and nuclear membranes [21,33] and high concentration of this compound inhibits the functional oxidase system and always induces LPO. As the reactive metabolite inducing LPO is believed to be the trichloromethyl radical that alters membrane function by blocking ion pumps within the cell [21], in our study also it appears that the PQQ inhibiting LPO might have been brought either through enhancing cytochrome P_{450} enzymes or through an inhibition in trichloromethyl radicals.

Our findings for the first time reveal that PQQ has the potential to ameliorate chemical induced hepatic LPO in three *in-vitro* systems. In fact, the radical scavenging capability of phenolic/ quinone compounds are due to their hydrogen donating ability or due to the number of hydroxyl groups present, which in turn modify the reactivity of the molecules [34-35].

In the present study the antiperoxidative role of PQQ is supported by the results of different antioxidant potential assays and is also compared to the some plant extracts and vitamin C, which are well known for their antioxidative nature [7,36]. In ABTS radical scavenging activity, the antioxidant capacity of PQQ, Vit.C and different plant extracts were evaluated according to the ABTS decolorization method. The results of antioxidant activity of all samples as expressed in percentage inhibition indicated that the PQQ displayed the highest radical scavenging potential in all doses as compared to Vit.C and all plant extracts, thus consolidating its better antioxidant potential as compared to Vit.C.

Somewhat similar findings were made with respect to scavenging the stable DPPH radical, a widely used method to evaluate the free radical scavenging ability of various samples [24]; DPPH is a stable nitrogen-centered free radical, the color of which changes from violet to yellow upon 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 [37]. Results of this study further confirmed that PQQ has high radical-scavenging activities with all doses as compared to Vit.C and all the test plant extracts.

Iron generates LPO by accelerating the dissociation of lipid hydroperoxides to their respective peroxy and alkoxy radicals [38]. In our study iron chelating percentage was highest for PQQ and it increased with increase in concentration. PQQ also showed higher metal chelating activity as compared to Vit.C and plant extracts (Table 4). These effects could be due to the presence of polyphenols which has potent iron chelating capacity. The reducing power of a PQQ is related to its electron transfer ability and may serve as a significant indicator of its potential antioxidant activity. With respect to superoxide

scavenging activity, it was observed that PQQ possesses better dose dependent superoxide scavenging potential than Vit.C and plant extracts. Probably this higher scavenging activity of PQQ is due to its high redox potential with hydroxyl group (O-H) that is easily liberated for stabilization of superoxide anion.

Hydrogen peroxide can cross cell membranes rapidly, once inside the cell, H_2O_2 may react with Fe^{2+}, and possibly Cu^{2+} ions to form hydroxyl radical and this may be the origin of many of its toxic effects [39]. Although GS extract showed better hydrogen peroxide scavenging activity at 25 and 50 µg/ml. At higher dose of PQQ appeared to be better than rest of all plant extracts and Vit.C. Whatever may be the mode of action(s), our findings clearly indicate that the antioxidative property of PQQ is better than the tested antioxidative plant extracts or Vit. C, suggesting that PQQ may prove to be beneficial to diseases associated with LPO. In conclusion, for the first time ameliorating effects of PQQ in the chemically ($FeSO_4$/ CCl_4/ H_2O_2)-induced *in vitro* LPO in hepatic tissue were evaluated. It is also emphasized that, as compared free radical scavenging potential of PQQ to some plant extracts, it contains higher antioxidative activities. Therefore, we suggest that the PQQ may be further studied to explore its therapeutic potential in treating different chronic diseases that are associated with LPO.

Acknowledgement

Financial support from the Council of Scientific and Industrial Research (CSIR), New Delhi, India. Ref. No. 09/301/(0121)/2011-EMR I for a Senior Research fellowship to Narendra Kumar is gratefully acknowledged.

Conflict of Interest

Authors do not have any conflict of interest.

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