



Evaluation of Free Radical Scavenging Potential of Pyrroloquinoline Quinone (PQQ): An *In Vitro* Study

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Abstract

In this investigation the free radical scavenging potential of pyrroloquinoline quinone (PQQ) was consolidated by *in-vitro* methods. 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 PQQ were evaluated in liver, the major target organ of a drug. 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. These effects were 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 were found most effective, suggesting their beneficial /antioxidative activity. The antioxidative potential of PQQ was thus affirmed by *in vitro* studies also. We suggest its therapeutic use, particularly in ameliorating oxidative stress associated diseases.

Keywords: PQQ; TBARS; CCl_4 ; H_2O_2 ; FeSO_4

Introduction

It is now well established 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 also a growing belief that most of the common health problems are associated with enhanced LPO [3-6]. Although few investigations are also there on the antiperoxidative effects of some plant extracts involving both *in vivo* and *in vitro* studies [7-11], nothing much is known on the antioxidative potential of non-vitamin antioxidants. Although pyrroloquinoline quinone (PQQ) is believed to be an antioxidant, it has not been well studied. 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 potential of PQQ after inducing the lipid peroxidation by 3 different chemicals. 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 from time to time [9,10,13,14]. Therefore, in this investigation the LPO was induced by FeSO_4 , H_2O_2 and CCl_4 and the efficacy tests were made considering inhibition of LPO in hepatic tissues by PQQ.

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

Thio-barbuteric acid (TBA) was procured from Hi-media pvt. Ltd. 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 [11,12,15].

Preparation of liver homogenate

For this, adult male rat were sacrificed after anaesthetizing with mild chloroform. Liver from

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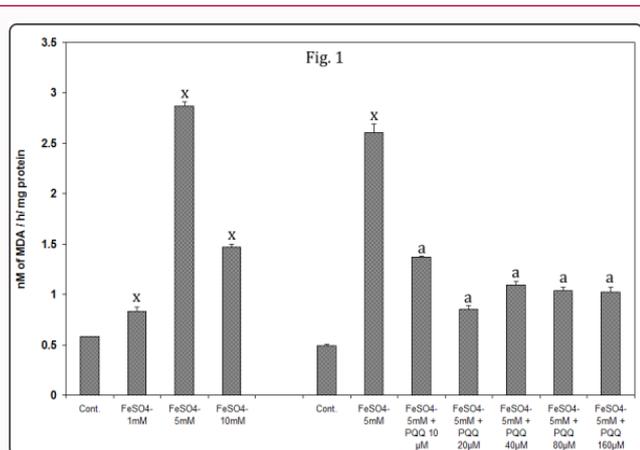


Figure 1: Effects of PQQ at 10, 20, 40, 80 and 160 μM on FeSO_4 -induced hepatic LPO. Data are mean \pm SEM (n=3). * $P < 0.001$ as compared to the respective control values. ^a $P < 0.001$ as compared to the respective FeSO_4 treated value.

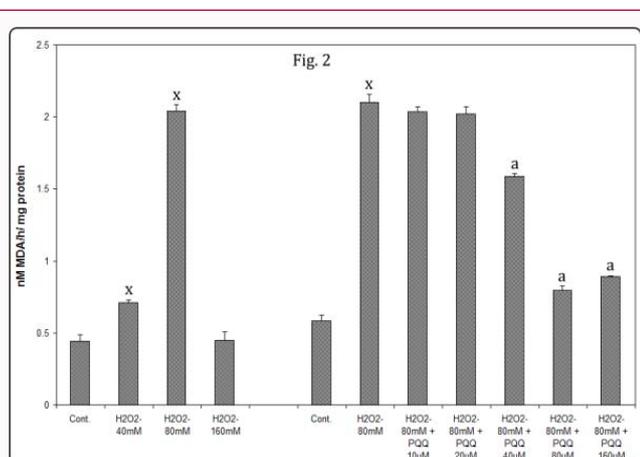


Figure 2: Effects of PQQ at 10, 20, 40, 80 and 160 μM on H_2O_2 -induced hepatic LPO. Data are mean \pm SEM (n=3). * $P < 0.001$ compared to the respective control values. ^a $P < 0.001$ as compared to the respective H_2O_2 treated value.

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 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. Two 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

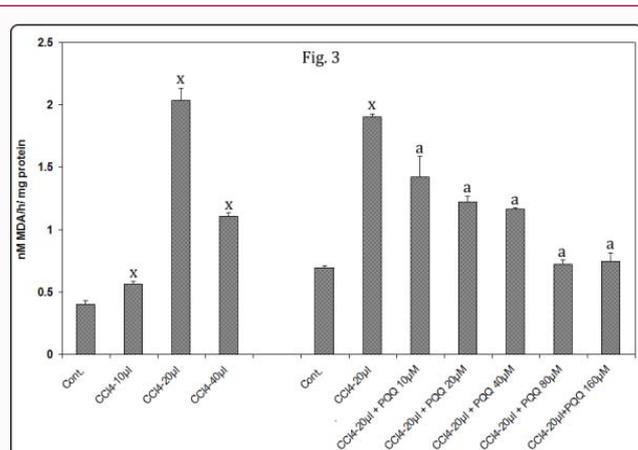


Figure 3: Effects of PQQ at 10, 20, 40, 80 and 160 μM on CCl_4 -induced hepatic LPO. Data are mean \pm SEM (n=3). * $P < 0.001$ as compared to the respective control values. ^a $P < 0.001$ as compared to the respective CCl_4 treated value.

liver homogenate, three different concentrations (1, 5 and 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. 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 in chopped liver 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 [11,16]. 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 Systems

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 reports [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. 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 , which 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, i.e. 10, 20, 40, 80 and 160 μM (each in triplicates). The mixture was incubated at 37°C for 2 h. Following the addition

of trichloroacetic acid (TCA) and TBA the optical density (OD) was measured at 532 nm [17]. 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, as mentioned above (each in triplicates). Following the incubation at 37 °C and the addition of TCA and TBA, OD was measured at 532 nm [18].

Statistical analysis

Data are expressed as mean \pm 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 (Figure 1). However, the maximum % of increase in LPO was observed at 5 mM of FeSO_4 (i.e. 394%). When considering this most effective concentration of FeSO_4 , antiperoxidative effect of the test compound, PQQ was evaluated at its five different concentrations, i.e. 10, 20, 40, 80 and 160 μM ; FeSO_4 -induced inhibition of LPO was observed 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.

Incubation of rat liver slices with different concentrations of H_2O_2 (40, 80 and 160 mM) also resulted in a significant increase in hepatic LPO (Figure 2). However, maximum percent increase i.e. 363% was observed at 80 mM. Considering this effective concentration of H_2O_2 to induce hepatic LPO, when incubated with any of the 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 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. 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 (Figure 3) in all three concentrations (40%, 407% and 175% respectively). Considering the effective concentration of CCl_4 that showed maximum increase in hepatic LPO (i.e. 20 μl), when 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 little 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.

Thus, concentration dependent effects were observed with PQQ that inhibited $\text{FeSO}_4/\text{CCl}_4/\text{H}_2\text{O}_2$ -induced hepatic LPO as maximum stimulation was observed in 5 mM, 20 μl and 80 mM of FeSO_4 , CCl_4 and H_2O_2 respectively. On using these concentrations when LPO inhibiting potential of PQQ was studied, out of its five different concentrations, only 20 μM , 80 μM and 80 μM were found to inhibit maximally the $\text{FeSO}_4/\text{CCl}_4/\text{H}_2\text{O}_2$ -induced tissue LPO respectively.

Discussion

The results 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 chemical oxidant was incubated with PQQ, concentration dependent effects were observed, but with all the concentrations, PQQ inhibited $\text{FeSO}_4/\text{CCl}_4/\text{H}_2\text{O}_2$ -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. 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 and H_2O_2 -induced tissue LPO respectively, suggesting that the best LPO inhibiting potential of PQQ can be obtained in a particular concentration.

As FeSO_4 -induced LPO is known to take place through ferryl per-ferryl complex [19] 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 possible 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 [9,11,20-22]. Interestingly, in all these cases, the chemical-induced LPO was inhibited by PQQ, again supporting its antiperoxidative nature.

H_2O_2 , a non-radical 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 [23], which in turn, induce peroxidation of lipids and proteins, affecting cell integrity [23,24]. Therefore, 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 [18,25] 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 [18], in our study appears that the PQQ inhibiting LPO might have been brought either through enhancing cytochrome P_{450} enzymes or through an inhibition in trichloromethyl radicals.

It may be emphasized that, our findings for the first time reveal the potential of PQQ 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 [26,27]. Whatever may be the mode of action(s), our findings for the first time clearly indicate the antioxidative property of PQQ in *in vitro*, consolidating its potential to scavenge free radicals and indicating that PQQ may prove to be beneficial in treating diseases associated with enhanced LPO. We suggest that the PQQ may be further studied to explore its therapeutic potential in treating different chronic diseases that are associated with LPO.

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