SHILAJIT DIBENZO-α-PYRONES: MITOCHONDRIA TARGETED ANTIOXIDANTS

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Summary

The decrease in efficiency of mitochondria in generating energy currency (ATP) in animals and in humans is associated with aging (geriatric problems) and oxidative stress. This deficiency has a link with the systemic deficiencies of coenzyme Q_{10} (CoQ_{10}) concentration and of two of its endogenous functional associates, namely, 3-hydroxydibenzo-α-pyrone (3-OH-DBP) and 3,8- dihydroxydibenzo-α-pyrone [3,8-$(OH)_2$-DBP]. Mitochondrial targeting of the two DBPs, isolated from shilajit (the supervitalizer of Ayurveda), and of CoQ_{10} could be formidable strategies to augment antioxidant defense and energy generating elements for restoring normal functions of mitochondria. DBPs, as also their fatty-acyl and amino-acyl conjugates, occur in animal mitochondria and in blood where they act in tandem with CoQ_{10} in the electron transport chain. Administration of CoQ_{10} alone, in mitochondrial deficiency states, therefore, could not restore normal mitochondrial functions. The concomitant targeting of DBPs and CoQ_{10} to mitochondria would augment energy (ATP) synthesis and protect redox states of CoQ_{10} from oxidative degradation. The present findings adduce evidence of augmentation of the concentrations of DBPs and CoQ_{10} in mitochondria when administered, from exogenous sources, through intra-peritoneal/oral route. Their probable mechanism of action would involve the three redox states of DBPs (reduced form, semiquinone radical and quinone form) and similar redox states of CoQ_{10} as a measure to restore normal energy synthesizing ability of mitochondria.

Keywords*:* Shilajit, hydroxylated dibenzo-α-pyrones, mitochondria, CoQ_{10}, ubiquinol stability

Introduction

Mitochondria are intracellular organelles responsible for energy metabolism. Consequently, mitochondrial dysfunction is damaging, particularly to neural and muscle tissues which have high energy demands. Mitochondrial dysfunction is central to a number of human degenerative diseases. Oxidative damage to the mitochondria is a major factor in the pathophysiology of Parkinson's disease, Friedreich's Ataxia and Wilson's disease, mtDNA disorders, diabetes, motor neuron disease and the non-specific loss of vigor associated with aging. Oxidative damage to mitochondria also contributes to the pathophysiology of inflammation and ischaemic-reperfusion injury in stroke, heart attack and during organ transplantation and surgery.

From the mid-1970s, CoQ_{10} was synthetically prepared and used as a therapeutic agent in human physiological deficiencies involving mitochondria. In the electron transport chain, according to the existing scientific tenet, CoQ_{10} receives electrons from NADH, forms reduced CoQ_{10} ($CoQH_2$) and passes the electron to cytochrome c, required for energy synthesis. After transfer of the electrons, $CoQH_2$ is again oxidized to the quinone form.

During oxidative stress, the oxidation-reduction cycle of $CoQH_2 \leftrightharpoons CoQ_{10}$ is impaired and CoQ_{10} is randomly degraded to form polar aberrant metabolites. Oxidation of $CoQH_2$ and degradation of subsequently formed CoQ_{10} by systemic oxygen-centered free radicals produces intractable agglomerates. Delivering CoQ_{10} alone, as was the common practice till now [1], could not restore normalcy from the systemic dysfunction. Researchers are persuasively indicating presence of some "unidentified" factors involved in mitochondrial energy transduction [2]. Accordingly, there is a need for compounds, compositions and methods that limit, prevent and/or restore from damage to mitochondria by targeting energy producing molecules to the organelle. Hydroxylated dibenzo-α-pyrones (DBPs) and their acyl and aminoacyl derivatives are the key bioactive components of shilajit. Shilajit is regarded in Ayurveda as the energy currency (supervitalizer) for living animals including humans. DBPs (and equivalents) are endogenously synthesized from PUFAs (EPA/DHA) and also occur in animal mitochondria and blood [3]. In a previous study, it was reported that shilajit acted as a potent antioxidant to protect mitochondria from oxidative damage and to resurrect its ATP producing potential [4]. The components of shilajit after reaching mitochondria exerted their: (i) reductive; (ii) $CoQH_2$ stabilizing and other attendant effects in ATP synthesis. The present study has been designed to obtain further evidence of targeting to mitochondria of two key bioactive DBPs of shilajit, namely 3-hydroxydibenzo-α-pyrone (3-OH-DBP) and 3,8- dihydroxydibenzo-α-pyrone [3,8-$(OH)_2$-DBP] [5]. Probable mechanism of action associated with this phenomenon has also been suggested.

Materials and Methods

Chemicals and Reagents: All chemicals and reagents were of analytical grade and obtained from Sigma-Aldrich, Merck, India or SRL, India. The solvents used for chromatographic techniques were of HPLC grade, obtained from Merck, India.

Test compounds: 3-OH-DBP and 3,8-$(OH)_2$-DBP were isolated from shilajit, and each purified to the extent of ≥ 99% following earlier described procedures [3,6]. Purity of the two DBPs was determined by HPLC using standard synthetic markers.

Antioxidant activity of DBPs

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay [7]: 0.5 ml DPPH solution (3 mg in 25 ml ethanol) was mixed with 0.5 ml sample solution and the absorbance of the mixture after 20 minutes of incubation in the dark was monitored at λ 517 nm. Upon reduction, the colour of the solution fades. The concentration that causes a decrease in the absorbance of initial DPPH radicals by 50% is defined as IC_{50} of the sample.

Superoxide ($O_2^{\cdot-}$) anion scavenging activity [7]: This assay was conducted following a published procedure. Briefly, $O_2^{\cdot-}$ was generated in solutions by the system consisting of NADH, PMS (phenazine methosulphate) and aerial O_2. NBT (nitroblue tatrazolium) was used as the probe. In presence of $O_2^{\cdot-}$, yellow colored NBT transformed into blue colored formazan which gives absorbance at λ 540 nm. Samples were mixed with the $O_2^{\cdot-}$ generating solution and the decrease in absorbance was monitored after proper incubation. The concentration that causes a decrease in the intensity of initial absorbance by 50% is defined as IC_{50} of the sample.

ABTS radical cation decolorization assay [7]: $ABTS^{\cdot+}$, the oxidant, was generated by persulfate oxidation of 2,2′-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid. This solution was diluted with phosphate buffer (pH 7.4) until the absorbance reached 0.7 to 0.8 at λ 734 nm. 1 ml of the resulting solution was mixed with the sample (0-20 µl). The absorbance was read at room temperature, 4 minutes after mixing. The concentration that causes a decrease in the absorbance of initial ABTS radicals by 50% is defined as IC_{50} of the sample.

Ferric reducing antioxidant potential, FRAP assay [7]: The oxidant in the FRAP assay was prepared by mixing TPTZ, acetate buffer and $FeCl_3.H_2O$ and referred to as "FRAP reagent". The final solution has Fe(III) of 1.67 mM and TPTZ of 0.83 mM concentration, To measure reduction potential, samples were added to FRAP reagent and the absorbances were noted at λ 593 nm after 30 minutes incubation at 37°C. The concentration that causes an increase in the intensity of color with respect to control (no sample) value by 50% is defined as IC_{50} of the sample.

Nitric Oxide Radical Scavenging Activity (NO assay) [7]: Nitric oxide (NO) radicals were generated from sodium nitroprusside solution in buffer saline and measured by Griess reagent. 1 ml of 10 mM sodium nitroprusside was mixed with 1 ml of sample with different concentrations in phosphate buffer (pH 7.4). The mixture was incubated at 25°C for 150 min. To1 ml of the incubated solution, 1 ml of Griess' reagent (1% sulphanilamide in 2% *o*-Phosphoric acid and 0.1% NEDA in 2% *o*-Phosphoric acid in 1:1 v/v ratio) was added and the absorbance was read at 546 nm. The concentration that causes a decrease in the intensity of color with respect to control (no sample) value by 50% is defined as IC_{50} of the sample.

Assay for inhibition of erythrocyte membrane Lipid Peroxidation [8]: The degree of lipid peroxidation was evaluated by estimating the thiobarbituric acid-reactive substances (TBARS) using a standard method after minor modifications. Briefly, different concentrations of the samples were added to rat erythrocyte suspension to a final volume of 900 µl and incubated for 5 min at 37°C. Lipid peroxidation was initiated by adding 100 µl of 15 mM $FeSO_4$ solution. After 30 min, 500 µl of this reaction mixture was placed in a tube containing 500 µl of 10% aqueous trichloroacetic acid (TCA), incubated for 10 minutes at room temperature and centrifuged at 3000 rpm. The supernatant was separated and mixed with 500 µl of 1% thiobarbituric acid (TBA) in 50% aqueous acetic acid.

The mixture was heated in a water bath at 85°C for 30 min to complete the reaction. The intensity of the pink coloured complex was measured at λ 535 nm. The concentration that caused a decrease in the TBARS level with respect to control (no sample) by 50% is defined as IC_{50}.

Animals: Male Swiss albino mice weighing 20-24 g and Sprague-Dawley rats were procured from Central Research Institute (Ayurveda), Govt. of India, Salt Lake City, Kolkata, were used. The animals were housed in an animal room with alternating light-dark cycle of 12 hr each. The animals were acclimatized for at least 7 days to the laboratory conditions before conducting experiments. Experiments were carried out between 0900 h and 1700 h. The study was conducted in accordance with Good Laboratory Practice (GLP) Regulations of WHO (WHO Document, 1998). The "Principles of laboratory animal care" (NIH Publication # 85-23, 1985) were followed in the study.

Treatment protocol – Mitochondrial targeting of the DBPs
Mice were divided into following two groups comprising 4 animals in each group.
Control: Received 0.8% (w/v) carboxymethyl cellulose (CMC) in water as vehicle intra-peritoneally.
DBPs: Received a 4:1 (w/w) mixture of 3,8-$(OH)_2$-DBP and 3-OH-DBP in CMC intra-peritoneally (20 mg/Kg body weight).
One hour after injection, the animals of both the groups were sacrificed and liver tissues collected.

Treatment protocol – Augmentation of total coenzyme Q by the DBPs in plasma and organs
Rats were divided into following two groups comprising 4 animals in each group.
Control: Received 0.8% (w/v) carboxymethyl cellulose (CMC) in water orally.
CoQ: Received CoQ_{10} in CMC orally (5 mg/Kg body weight).
CoQ+DBPs: Received 3,8-$(OH)_2$-DBP in CMC orally (5 mg/Kg body weight).
The drugs were administered to the animals once per day for 5 days. Animals were subjected to hypoxic stress for 1 hour each day for 3 days from the 3rd day of the experiment. After the stipulated time-period, the content of total coenzyme Q were estimated in heart, kidney, liver (in wet weight basis) and in blood, by HPLC, after proper extraction from the tissues according to a published procedure [9].

Extraction and estimation of DBPs from liver mitochondria: Mitochondria were isolated from liver following a published procedure [10]. Briefly, liver tissues were triturated with sterilized medium comprising 0.25 M sucrose, 1 M EDTA, 10 mM Tris-HCl; pH 7.4. The homogenates were centrifuged at 900g for 20 minutes. The sediment was discarded and the trituration step was repeated again. After centrifugation, the clear supernatant was centrifuged twice at 12,000g, 20 minutes each time, to form pellets of a mitochondria-enriched fraction. The pellets were extracted with the Bligh and Dyer solvent system and the extracts were subjected to comprehensive HPTLC analyses for detection and quantitation of DBPs.

Stabilization of $CoQH_2$ *in vitro* by 3,8-$(OH)_2$-DBP at different pH: 2 mg of solid CoQ_{10}, in an airtight vial, was dissolved in 1 ml of methanol. After thorough vortexing, 2 mg of solid $NaBH_4$ was added, 1 mg at a time, to the yellow colored solution and kept in an ice-bath for a few minutes. The vial was kept closed to avoid contact with air but opened occasionally to avoid generation of pressure inside due to accumulation of hydrogen.

After completion of reduction, a clear, colorless solution was obtained to which methanolic solution of 3,8-$(OH)_2$-DBP (1 mg/ml) was added in a ratio of 1:1 (v/v) to obtain a final concentration of the mixture – $CoQH_2$ 1 mg/ml and 3,8-$(OH)_2$-DBP 0.5 mg/ml. This solution (pH 8) was analyzed by HPLC for $CoQH_2$ content at different time intervals. A control $CoQH_2$ solution was prepared in a similar fashion except that no 3,8-$(OH)_2$-DBP was added. To obtain solutions of $CoQH_2$ with pH 7 and pH 3, aliquots of concentrated HCl were added to the methanolic solution of $CoQH_2$ of pH 8. The results are expressed as average of two analyses (Tables 5-7).

Chromatographic Techniques

HPTLC Conditions: HPTLC was performed on Merck KGaA (1.05554.0007) pre-coated silica gel 60 F_{254} aluminium TLC plates. Solutions of marker 3-OH-DBP and 3,8-$(OH)_2$-DBP, along with experimental samples were applied and the plates were developed in a twin trough chamber with chloroform:methanol 9:1 (v/v) as mobile phase. Densitometric evaluation of the plates was performed at λ 240 nm by means of Camag TLC Scanner 3 in absorption mode. The scanned data were processed by means of Camag winCATS software, version 1.3.4. The plates were subsequently scanned to determine the UV reflectance spectra of each spot between 200 and 400 nm to identify the two bioactives of shilajit.

HPLC conditions for DBP analyses: HPLC analysis of DBPs were performed using Waters HPLC system comprised with PDA detector and Empower software with a NovaPak pre-packed column (RP C_{18}, particle size 4 µm, 3.9 x 150 mm) fitted with a reverse phase guard column and acetonitrile:water:*o*-phosphoric acid 32:67:1 (v/v/v) as mobile phase, with a run time of 15 minutes and flow rate 1 ml/min in an isocratic mode. Detection was done at λ 240 nm.

HPLC conditions for CoQ_{10} and $CoQH_2$ analyses: HPLC analysis of CoQ_{10} and $CoQH_2$ were performed using Waters HPLC system comprised with PDA detector and Empower software with a Xterra pre-packed column (RP C_{18}, particle size 5 µm, 4.6 x 250 mm) fitted with a reverse phase guard column and methanol:acetonitrile:ethanol 30:30:40 (v/v/v) as mobile phase, with a run time of 15 minutes and flow rate 1 ml/min in an isocratic mode. Detection was done at λ 275 nm for CoQ_{10} and 290 nm for $CoQH_2$.

Results

Purity of DBPs used in the present study: Purity of the test DBPs was determined by HPLC using respective standard DBPs as marker and was found to be ≥99% (Fig. 1).

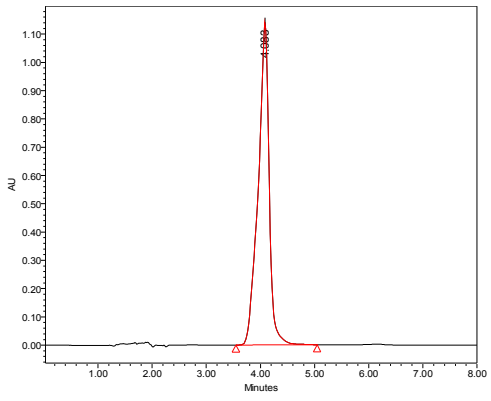


Fig. 1A – HPLC chromatogram of test 3,8-$(OH)_2$-DBP (used in this study)

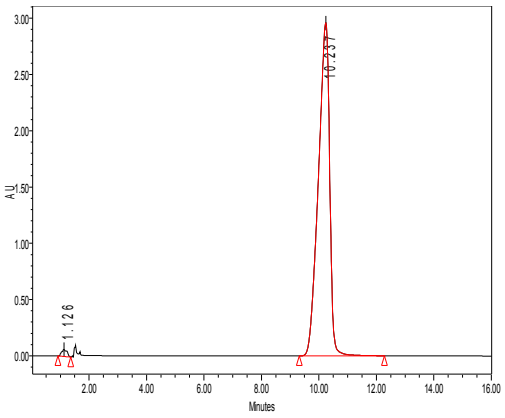


Fig. 1B – HPLC chromatogram of test 3-OH-DBP (used in this study)

Antioxidant activity of DBPs: Antioxidant profile of the two test DBPs is furnished in Table 1. The results suggest that both the DBPs possess potent radical scavenging activity, while 3,8-$(OH)_2$-DBP acts better than 3-OH-DBP in this aspect.

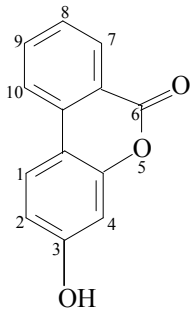
Table 1. Comparative *in vitro* antioxidant potency of the two test DBPs. The results are given in IC_{50} values of the substances.

Assay methods	3-OH-DBP (µM)	3,8-$(OH)_2$-DBP (µM)
DPPH radical scavenging assay	-	0.54±0.03
ABTS radical scavenging assay	0.98±0.05	0.03±0.01
FRAP assay	0.41±0.03	0.08±0.01
$O_2^{\cdot-}$ scavenging assay	0.43±0.06	1.21±0.15
NO assay	3.27±0.13	5.09±0.22
Inhibition of lipid peroxidation	0.30±0.08	0.07±0.01

Estimation of DBPs in mice liver mitochondria after administration of the test DBPs: 3-OH-DBP, 3,8-$(OH)_2$-DBP and their redox products [3] were detected and quantitated in mitochondria of control and test compounds treated mice by HPTLC (Table 2 and structures I-IV).

Table 2. HPTLC reflectance spectral data of oxido-reductive products of 3-OH-DBP and 3,8-$(OH)_2$-DBP (formed *in vivo*) after treatment with the test compounds.

Product	R_f	Reflectance spectrum: λ_{max} (nm)
3-OH –DBP (I)	0.65	235, 277, 300, 335
3,8-$(OH)_2$-DBP (II)	0.59	220, 235, 280, 304, 355
3,8-$(OH)_2$-DBP-semiquinone (III)	0.52	225, 238, 280, 304, 336, 357, 370
3,8-$(OH)_2$-DBP-quinone (IV)	0.43	234, 286



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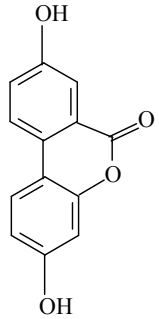

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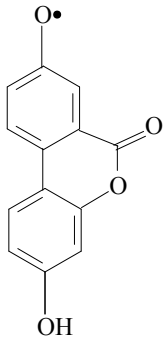

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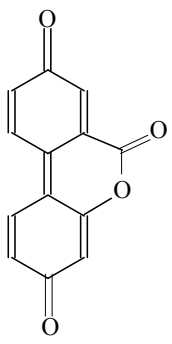

IV

Intra-peritoneal administration of DBPs to the animals increased the mitochondrial amounts of DBPs. The amounts of redox products of 3,8-$(OH)_2$-DBP isolated form mice liver mitochondria are given in Table 3. Since 3-OH-DBP is systemically converted into 3,8-$(OH)_2$-DBP, this data include the contribution of the administered 3-OH-DBP.

Table 3. Amounts of DBP-products in mitochondria of mice liver before and after treatment (i.p.) with DBPs, analyzed by HPTLC

Compounds	Control mitochondria (nmol/gm wet wt)	DBP treated mitochondria (nmol/gm wet wt)
3,8-$(OH)_2$-DBP (II)	0.5±0.02	2.3±0.08
3,8-$(OH)_2$-DBP-semiquinone (III)	12.7±0.15	20.2±0.22
3,8-$(OH)_2$-DBP-quinone (IV)	26.7±0.65	24.8±0.58

Augmentation of coenzyme Q in mouse organs by 3,8-$(OH)_2$-DBP: Co-administration (p.o.) of CoQ_{10} with 3,8-$(OH)_2$-DBP significantly improved the contents of total coenzyme Q (CoQ_9 and CoQ_{10}) in different organs, except in plasma. The results of HPLC determinations are incorporated in Table 4.

Table 4. Increment of total coenzyme Q after co-administration of CoQ_{10} and 3,8-$(OH)_2$-DBP in rat blood plasma and organs

Treatment/ Groups	Amounts of total CoQ_9 and CoQ_{10}			
	Plasma (nmol/ml)	Heart (nmol/gm)	Liver (nmol/gm)	Kidney (nmol/gm)
Control	0.79±0.03	18.46±0.55	15.38±0.48	11.86±0.14
CoQ_{10}	0.81±0.02	35.69±0.86	19.41±0.38	16.51±0.09
CoQ_{10}+DBPs	0.82±0.03	36.99±0.89	25.03±0.55	17.81±0.13

Maintenance of reduced state of CoQ_{10} by 3,8-$(OH)_2$-DBP at different pH: Maintenance of the stability of $CoQH_2$ by 3,8-$(OH)_2$-DBP at alkaline, neutral or acidic pH, with time, was noted (Tables 5-7). 3-OH-DBP *per se* did not provide protective umbrella to the reduced state of CoQ_{10} to any significant extent.

Table 5. Stability of $CoQH_2$ in absence or presence of 3,8-$(OH)_2$-DBP at pH 8

Time (min)	Residual $CoQH_2$ (%)in absence of 3,8-$(OH)_2$-DBP	Residual $CoQH_2$ (%) in presence of 3,8-$(OH)_2$-DBP
0	100.00	100.00
40	79.18	93.44
60	54.09	80.21
80	33.17	66.57
100	20.87	51.85

Table 6. Stability of $CoQH_2$ in absence or presence of 3,8-$(OH)_2$-DBP at pH 7

Time (hour)	Residual $CoQH_2$ (%) in absence of 3,8-$(OH)_2$-DBP	Residual $CoQH_2$ (%) in presence of 3,8-$(OH)_2$-DBP
0	100.00	100.00
48	7.73	10.79
72	3.70	6.33

Table 7. Stability of $CoQH_2$ in absence or presence of 3,8-$(OH)_2$-DBP at pH 3

Time (hour)	Residual $CoQH_2$ (%) in absence of 3,8-$(OH)_2$-DBP	Residual $CoQH_2$ (%) in presence of 3,8-$(OH)_2$-DBP
0	100.00	100.00
48	57.25	79.27
72	61.95	73.91

Discussion

The key bioactives of shilajit, namely, 3-OH-DBP and 3,8-$(OH)_2$-DBP, were highly antioxidative in nature as was observed by the conventional *in vitro* radical scavenging assays (Table 1). Since, shilajit was found to augment systemic ATP synthesis in debilitating physiological conditions [4], DBPs were considered to play a key role in such activity. This would require their targeting to mitochondria, when administered from exogenous sources. Administration (i.p.) of DBPs to mice showed not only the presence of DBPs and their incipient redox products (semiquinone radical and quinone) in the mitochondria (Table 2, determined by HPTLC) but also showed increment vis-à-vis control (Table 3). These observations suggest that the administered DBPs can augment their contents in the mitochondria that might play a crucial role in improved functioning of the electron transport chain.

CoQ_{10} is present in all tissues and membranes in highly variable amounts. By interaction with NADH, CoQ_{10} is converted into $CoQH_2$, which plays the electron-carrier role in the mitochondrial electron transport chain. $CoQH_2$ is also a highly efficient antioxidant in preventing lipid, protein and DNA-oxidative damage and is continuously regenerated from CoQ_{10} by intracellular reduction systems. In some pathologic processes, when tissue concentration of CoQ_{10} is decreased, it may be advantageous to supplement CoQ through dietary substances [11]. However, the effect of administration of CoQ_{10} by dietary supplement is difficult to interpret because loose CoQ_{10} might also act as a pro-oxidant [12]. Improvement in the energy generating process in animals by shilajit [4] indicates that DBPs might act in tandem with $CoQ_{10}/CoQH_2$ in mitochondria. *In vitro* experiment revealed that 3,8-$(OH)_2$-DBP converted CoQ_{10} to the semiquinone radical and stabilized the latter by spin-pairing (data not shown). The preservation of the reduced form of the coenzyme seems to be an attendant function of DBPs. This was simulated in the *in vitro* experiment of stabilization of $CoQH_2$ by 3,8-$(OH)_2$-DBP (Tables 5-7) at different pH conditions. The comprehensive role of DBPs, in association with CoQ_{10}, in the electron transport chain, is now being investigated.

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