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Reduction of oxidative stress induced vanadium toxicity by complexing with a flavonoid, quercetin: A pragmatic therapeutic approach for diabetes

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Abstract

Vanadium compounds are known to lower blood glucose level in diabetes but are associated with toxicity. *In vitro* cytotoxicity of $VOSO_4$ and bis(quercetinato) oxovanadium(IV) (BQOV) was examined in CHO cells. Both the agents showed time and dose dependent increase in ROS generation however it was relatively less in BQOV. Moreover, $VOSO_4$ also caused higher necrosis. Hypoglycemic potential of $VOSO_4$ and BQOV was tested in streptozotocin-induced diabetic Balb/c mice. A marked difference was observed in the hypoglycemic action of $VOSO_4$ and BQOV treated mice that lasted only for about 6 h in $VOSO_4$ as against 24 h in BQOV. Comparison of acute toxicity of the compounds in normal Balb/c mice revealed negligible nephrotoxicity of BQOV. Kidney analyses of $VOSO_4$ treated animals' revealed high ROS generation and tubular necrosis. Similarly serum levels of urea and creatinine were elevated in these animals indicating kidney dysfunction. No such abnormality was observed in BQOV treated animals. Reduced nephrotoxicity of BQOV could be due to increased catalase activity found in the kidney of BQOV treated animals and BQOV's radical scavenging activity. The data clearly demonstrates immense hypoglycemic activity and reduced toxicity of BQOV thus making the conjugate a suitable candidate for therapeutic utility.

Abbreviations: CHO – Chinese Hamster Ovary; DMEM – Dulbecco's Modified Eagle Medium; DMSO – dimethyl sulfoxide; ROS – Reactive Oxygen Species; DCFH-DA – *2′,7′-dichlorofluorescin diacetate*.

Introduction

Diabetes mellitus is a chronic, metabolic disorder wherein lack of insulin secretion (type 1) and/or increased cellular resistance to insulin (type 2) results in disturbance of carbohydrate, protein and lipid metabolism. Hence, it can affect nearly every organ system in the body. Current treatment regime includes daily insulin injections in case of type 1 diabetes while treatment of type 2 diabetes includes a number of oral hypoglycemic agents (sulfonylureas, biguanides, troglitazones, etc) both individually as well as in a combination therapy, depending upon the individual need to attain normoglycemia. However these treatments fail to achieve stringent metabolic control in more than 50% of type 2 diabetics (Williams 1994). This limitation demands search for novel antidiabetic agents that mimic or enhance the properties of insulin as well as protect against diabetic complications.

Vanadium has been proved to be a potential antidiabetic agent since 1980s but hitherto it has not reached the clinics (Heylinger et al. 1985). Vanadium compounds have been found useful in case of both type 1 and type 2 diabetes as it can

mimic as well as enhance the activity of insulin both *in vivo* and *in vitro* (Verma et al. 1998). Several animal and human studies have demonstrated it's usefulness in treatment of diabetes still their clinical use has not been advocated due to associated toxicity of the metal (Srivastava, 2000). Vanadium has been proved to be toxic to cells *in vitro* and its toxicity has been attributed to its prooxidant nature (Cortizo *et al*. 2000; Zhang *et al*. 2006). Being a transition metal element, vanadium may participate in reactions involving formation of free radicals (Crans *et al*. 2004). Shi *et al*. (1996) have shown that in vitro incubation of vanadium (IV) with molecular oxygen dependent 2′-deoxyguanosine or with DNA in presence of H_2O_2 results in enhanced 8-hydroxy-2′dG formation and substantial DNA strand breaks. Moreover, vanadium compounds activate many key effector proteins of the signaling pathways including AP-1, MEK-1, ERK, JNK-1, PI-3 K and NF-κB. Activation of these pathways is also linked to the formation of ROS and DNA damage (Chen *et al*. 1999).

Animal studies revealed various toxic effects induced by vanadium compounds. Acute toxicity of vanadium involves dehydration, reduction of body weight, loss of appetite, renal tubular necrosis and pulmonary hemorrhage (Domingo 2002; Valko *et al*. 2005). Intraperitoneal injections of rats with orthovanadate revealed nephrotoxicity (Ciranni *et al*. 1995). Moreover, effects of vanadium on the reproductive and developmental functions have also been well established (Morgan & El-Tawil 2003).

Since inorganic vanadium is poorly absorbed from the GI tract and some GI difficulties have been reported various researchers have synthesized a number of organic vanadium compounds. Conjugation with an organic moiety increases bioavailability of vanadium (Thompson 1999). One of the challenges of designing metal-based drugs is to balance the potential toxicity of an active formulation with the substantial positive impact of the therapeutic aid (Thompson and Orvig 2003). Recently several researchers have taken up a combinatorial approach wherein they combine vanadium treatment regime with another antidiabetic agent like Trigonella foenum graecum (Preet *et al*. 2005) or antioxidant like black tea decoction (Clark *et al*. 2004) so as to reduce its toxicity without compromising its antidiabetic potential. In order to circumvent the aforementioned ill effects of vanadium we have synthesized vanadium–quercetin conjugate (bis(quercetinato)oxo vanadium (IV), BQOV) ($C_{32}H_{30}O_{24}SV_2$) which has been proved to be an effective oral hypoglycemic agent (Shukla *et al*. 2004). Further we have hypothesized that quercetin (a strong antioxidant) would combat prooxidant nature of vanadium and make BQOV much less toxic than its parent vanadium salt, $VOSO_4$. In this paper we have compared BQOV's cytotoxicity with $VOSO_4$ *in vitro* and acute toxicity of the two compounds *in vivo*. Our study indicated that BQOV is less toxic and more hypoglycemic than $VOSO_4$.

Methods

Cell culture and vanadium treatment

CHO cell line was used in this study. Cells were cultured in 25 cm^2 flasks in DMEM with 10% fetal bovine serum (FBS) and incubated in a humidified atmosphere of 5% CO_2 in air at 37 °C. BQOV was dissolved in DMSO and $VOSO_4$ was dissolved in deionized water at a stock concentration of 50 mM. Treatment of the compounds was performed in serum free medium.

Oxidative stress (ROS generation) assessment

5×10^3 cells/well were seeded in a 96 well plate and allowed to attach and grow for 24 h. The cells were then treated with the two compounds at different doses of uncomplexed vanadium (100 and 200 μM) for different time intervals (1, 24 and 48 h). ROS generation due to the treatment was determined by DCFH-DA staining (Ye *et al*. 1999). Briefly, the dye was added in the culture medium at a final concentration of 10 μM for 30 min and then fluorescent intensity of the cells was measured using fluroscan (Fluoroskan Ascent FL, Thermo Electron Corp.) by excitation at 485 nm and detection at 538 nm.

Cytotoxicity assessment

Cells were treated with the vanadium compounds and then fixed with 70% ethanol, treated with RNaseA followed by propidium iodide staining for cell cycle analysis using FACScan (Becton Dickenson, CA). Further, DNA fragmentation

assay was done on the cells. Briefly, cells were washed with calcium, magnesium free HBSS containing 0.02% EDTA and then lysed in TE lysis buffer containg 0.25% triton-X-100 followed by RNaseA and proteinase K digestion. Finally loading dye was added in the cleared solution and run on 1.5% agarose gel (Park & Patek 1998).

Hypoglycemic potential assessment

Mice were made diabetic by streptozotocin injection (200 mg/kg body weight, i.p.) and mice showing blood glucose above 200 mg/dl were taken for hypoglycemic studies. Diabetic mice were randomly divided into three groups each having 5–6 animals: one group injected intraperitoneally with $VOSO_4$ and other with BQOV at a dose of 0.1 mmol elemental V/kg body weight, the third set was kept as untreated diabetic control. Blood glucose level was determined just prior to and at regular intervals after injection up to 24 h, by withdrawing blood by tail nick method and using an automated glucose analyzer (Accu-Check sensor comfort, Roche Diagnostics, Germany). Balb/c mice (6–8 weeks) were obtained from the Animal Facility of National Centre for Cell Science, Pune, India. During the whole experimentation period mice were kept under controlled conditions (22 ± 2 °C) with 12 h light and dark cycles and had free access to water and feed. All animal experiments were performed according to the guidelines approved by the committee for the purpose of control and supervision of experiments on animals (Government of India) and with the permission of the institute's animal care and use committee.

Acute toxicity testing

LD_{50} of $VOSO_4$ has been reported to be 113 mg/kg when administered intraperitoneally in mice (Llobet & Domingo 1984), however in our laboratory we have found it to be 90 mg/kg i.e. 0.35 mmol vanadium/ kg body weight in case of normal Balb/c mice. Hence, to evaluate acute toxicity of BQOV, normal mice were injected intraperitoneally with 0.35 mmol/kg body weight of elemental vanadium in the form of $VOSO_4$ and BQOV and were observed initially for behavioral changes and then sacrificed after 12 h to evaluate its effect on kidney by serum analysis and histopathological examination (hematoxylin & eosin staining) of kidney.

Oxidative stress and antioxidant status of kidney

Amount of ROS generated in the kidney due to injection of the compounds was estimated by DCFH-DA staining (Koya *et al*. 2003). Briefly, 12 h after injection tissue was excised and weighed, and then it was incubated with the dye at a final concentration of 10 μM for 30 min at 37 °C, finally total fluorescent intensity of the tissue was measured using fluroscan by excitation at 485 nm and detection at 538 nm. Moreover to evaluate antioxidant status of kidney relative amount of SOD and activity of catalase was determined in different animal groups. Briefly, tissue was excised and homogenate was prepared in Tris buffer (pH 7.4) and stored at −85 °C until the assay. Amount of SOD was determined by SOD ELISA kit (Calbiochem) and catalase enzyme activity was determined spectrophotometricaly by evaluating amount of H_2O_2 degradation at 240 nm. The enzyme activity is expressed as unit (U), where 1 U is equal to amount required degrading 1 μmol of H_2O_2/minute/mg protein.

Antioxidant potential assessment

The SOD activity of the compounds was determined spectrophotometricaly by the NBT assay employing Xanthine/Xanthine oxidase as the source of superoxide radical (Dutta *et al*. 2001). One unit of SOD activity was defined as the concentration of the test substance required for 50% inhibition of NBT reduction by the superoxide (IC_{50}) anion. Moreover, Ferric reducing antioxidant power (FRAP) assay was performed (Benzie & Strain 1996). Briefly, FRAP values were obtained by comparing the absorbance change due to ferric to ferrous ion reduction at 593 nm in test reaction mixtures with those containing ferrous ions in known concentration.

General

CHO cells were obtained from National Centre for Cell Science, Pune, India. 25 cm^2 flasks and 96 well plates were purchased from NUNC. DMEM and FBS were purchased from Gibco. Fine chemicals

like quercetin, streptozotocin, DCFH-DA, propidium iodide, DMSO, RNaseA and proteinase K were purchased from Sigma. $VOSO_4$ was purchased from Thomas Baker while BQOV was prepared according to the method described earlier (Shukla *et al*. 2004).

Statistics and data analysis

Data are expressed as mean ± SD. Statistical significance of the difference in the means in two different groups was assessed by ANOVA. P value of less than 0.05 was considered significant.

Results

Oxidative stress and cytotoxicity

CHO cells were treated with the agents at different concentrations for various time intervals and then stained with DCFH-DA to measure amount of ROS in the cells and with PI to determine cell death. Figure 1 clearly demonstrates time and dose dependent increase in ROS generation in the cells by both the compounds. However, the amount of ROS generated by BQOV is negligible compared to the vanadium salt, $VOSO_4$. Figure 2a shows the cell cycle analysis of PI stained cells and demonstrates time and dose dependent increase in cell death in $VOSO_4$ while BQOV treated cells showed negligible cell death compared to $VOSO_4$ at any given time or concentration. This was further confirmed by DNA fragmentation assay, wherein $VOSO_4$ treated cells demonstrated a clear increase in low molecular weight (< 1 Kbp) DNA fragments which were not visible in BQOV treated cells (Figure 2b).

Hypoglycemic potential

It is clearly seen from Figure 3 that vanadyl sulfate lowers blood glucose level after 3 h of administration but the glucose level starts rising from 6 h onwards whereas BQOV normalizes the blood glucose and its effect last for about 24 h.

Acute toxicity

After intraperitoneal administration of $VOSO_4$ at a dose of 0.35 mmol V/kg body weight mice had shown immediate signs of acute toxicity such as

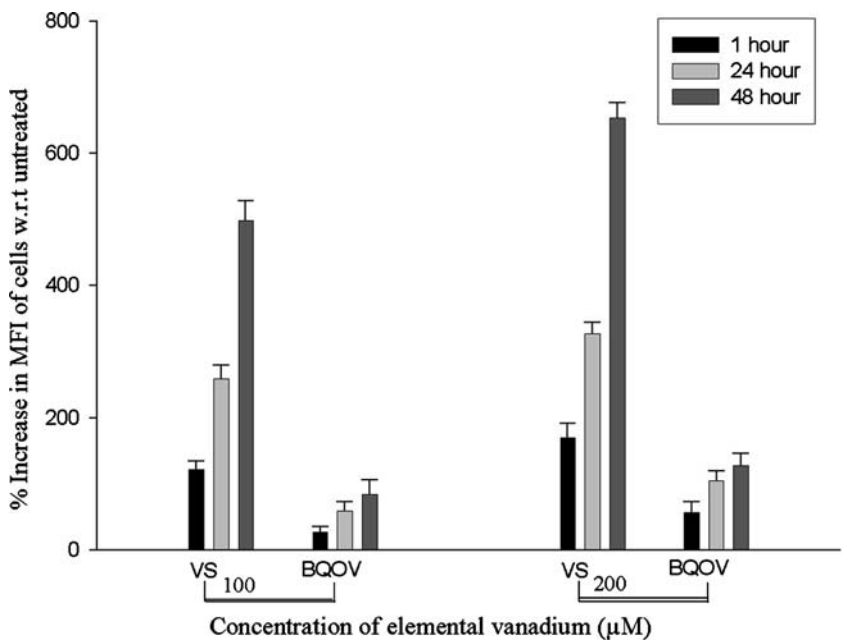

Figure 1. Graph represents relative amount of ROS generated in CHO cells by $VOSO_4$ (VS) and BQOV treatment. CHO cells were seeded in a 96-well-plate and allowed to attach and grow for 24 h. The cells were then treated with these vanadium compounds for different time intervals and amount of ROS generated at the end of the treatment was determined by DCFH-DA staining. Bars represent percent increase in mean fluorescent intensity (MFI) of cells with respect to untreated cells. Data are mean ± SD.

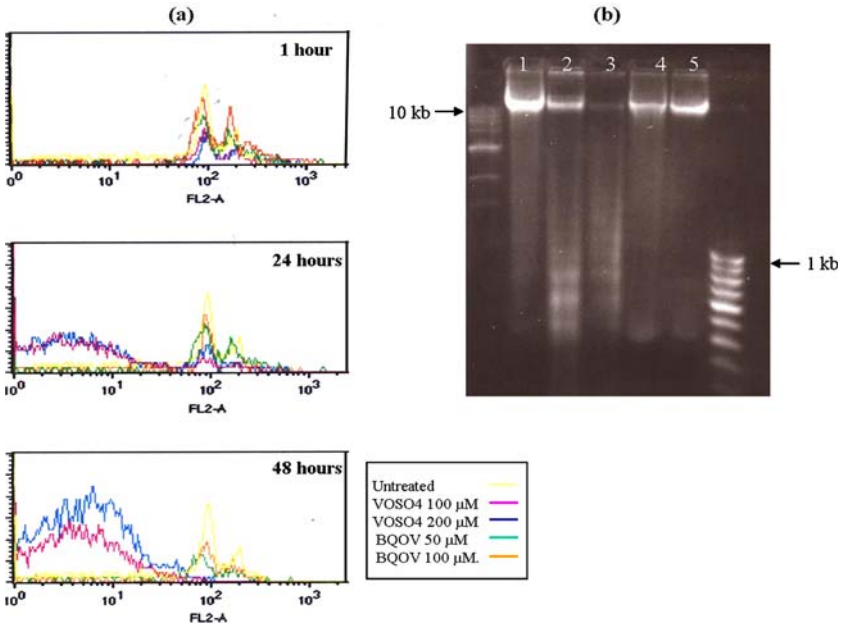

Figure 2. (a) Represents cell cycle analysis of PI stained CHO cells at 1, 24 and 48 h after treatment with the agents (b) Shows DNA fragmentation in various groups 24 h after treatment: untreated (1), $VOSO_4$ 100 μM (2), $VOSO_4$ 200 μM (3), BQOV 50 μM (4), BQOV 100 μM (5).

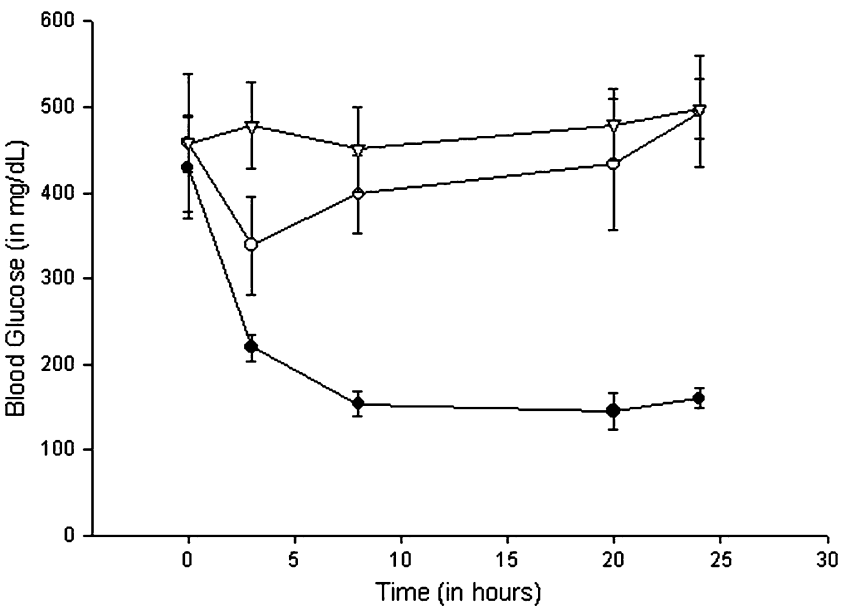

Figure 3. Comparative graph of blood glucose level of an untreated diabetic mice and changes in blood glucose level upon intraperitoneal administration of $VOSO_4$ (-°-) and BQOV (-●-) at a dose of 0.1 mmol V/kg body weight. Values represent mean ± SD.

irregular respiration, diarrhea and general lethargy further leading to paralysis of the hind limbs. However when mice were injected with BQOV there were no signs of toxicity.

Serum was collected 12 h post injection of either $VOSO_4$ or BQOV and serum creatinine and urea levels were determined in order to check kidney functionality. As evident from

Figure 4a serum creatinine and urea levels were elevated in $VOSO_4$ injected animals indicating kidney dysfunction, whereas there was no significant difference between BQOV injected and untreated animals. Moreover histopathological analysis of kidneys from $VOSO_4$ injected animals exhibited signs of acute tubular necrosis whereas there was no abnormality in BQOV injected animals (Figure 4b). Moreover if animals were kept for longer duration (around 24 h) contrary to $VOSO_4$ where 50% animals die, in case of BQOV no lethality was observed.

Oxidative stress and antioxidant status of kidney

Table 1 depicts the relative amount of ROS generated and antioxidant status in the kidney 12 h after treatment. It is observed that there was about 2-fold increase in amount of ROS after $VOSO_4$ injection while there was no oxidative stress in case of BQOV. Further, there was no difference in the relative amount of Zn–Mn SOD enzyme in various groups while there was an increase in catalase activity in case of BQOV group.

Antioxidant capacity of the compounds

SOD IC_{50} of quercetin and BQOV were found to be 0.58 mM and 0.63 mM, respectively, while it was 4.1 mM in case of $VOSO_4$. FRAP value/4 min/100 mM compound was found to be 5.43 for quercetin, 4.88 for BQOV and nil for $VOSO_4$. Hence it seems that BQOV has equivalent antioxidant capacity as that of quercetin.

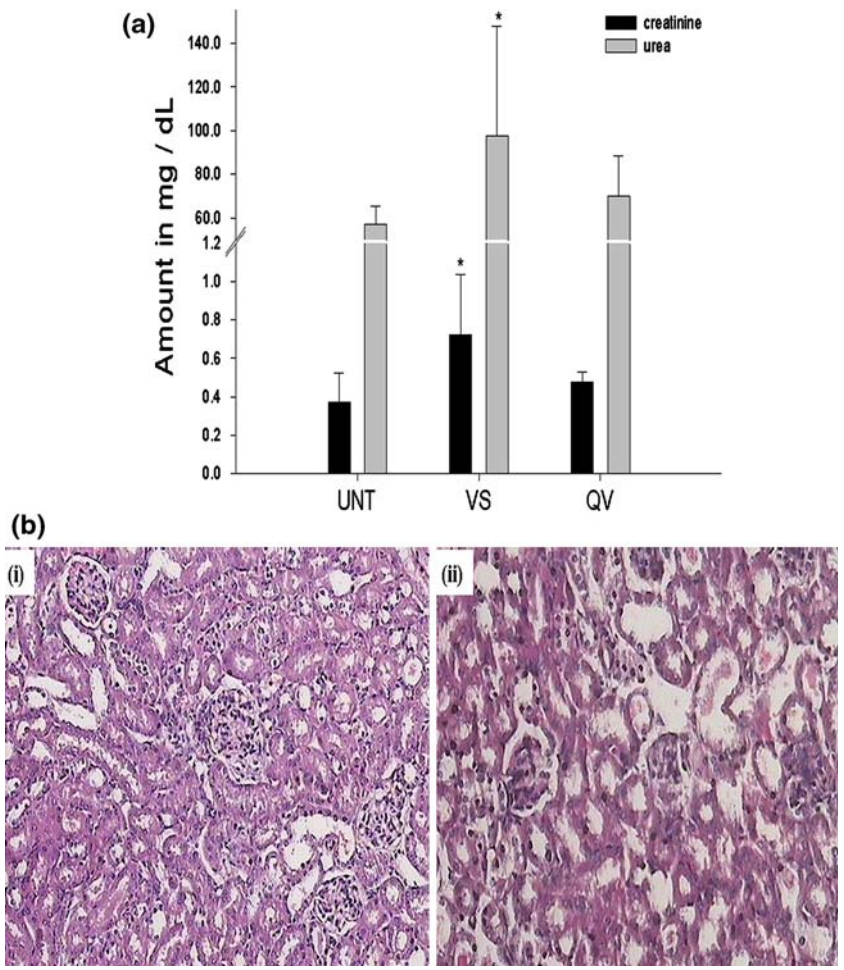

Figure 4. (a) Represents serum urea and creatinine levels 12 h after treatment with $VOSO_4$ (VS) and BQOV (QV). Values represent mean ± SD, $n = 5$, $*p < 0.05$ vs. untreated. (b) Represents H & E stained kidney sections of (i) untreated and (ii) $VOSO_4$ treated.

Table 1. Kidney was excised 12 h after treatment.

Group	MFI/100 mg tissue		Catalase (U)		SOD (ng/mg protein)	
	Mean	SD	Mean	SD	Mean	SD
Untreated	13.19	1.21	8.36	1.14	6.44	1.73
$VOSO_4$	29.88**	1.53	8.48	1.68	5.36	1.28
BQOV	14.33	1.8	11.92*	2.66	5.77	2.13

Total ROS in the tissue was then determined by DCFH-DA staining and Zn–Mn SOD level was determined by ELISA. Further catalase activity was determined spectrophotometricaly where U is μmol/min/mg protein. Values represent mean ± SD *$p < 0.05$ vs. untreated, **$p < 0.001$ vs. untreated.

Discussion

Vanadium is known to have hypoglycemic activity but its use is limited due to strong prooxidant nature. Hence research is still underway to balance between therapeutic potential and associated toxicity of the metal. In the present investigation we have demonstrated that prooxidant nature of vanadium could be counteracted by conjugating it with quercetin, thus makes it a viable option for therapeutic utility. Quercetin exhibits various remarkable array of pharmacological and biochemical actions like anti-viral, anti-cancer and strong antioxidant activity (Bhattaram *et al.* 2002). Moreover, quercetin is useful in preventing diabetic complications like retinopathy as it acts as an aldose reducatase inhibitor (Chaudhry *et al.* 1983) and overall oxidative stress of the body during diabetes (Mahesh *et al.* 2004).

We have previously reported the immense hypoglycemic potential of BQOV compared to $VOSO_4$ when administered orally to STZ-diabetic mice (Shukla *et al.* 2004). Here we found that vanadyl sulfate has a very short-term effect on blood glucose level when administered intraperitoneally. This result is in agreement with other studies wherein $VOSO_4$ is given at the same dose and by similar route (Yuen *et al.* 1995). It is interesting to observe that when quercetin is conjugated with vanadium it showed a prolonged hypoglycemic effect as euglycemic status is maintained until 24 h. When vanadium is conjugated with maltol i.e. in case of BMOV very similar effect has been observed (Yuen *et al.* 1995) hence, we claim that like maltol, quercetin is also acting as an organic ligand and improved bioavailability of vanadium.

In vitro vanadium has been shown to produce high oxidative stress in the cells as it produces H_2O_2 (Krejsa *et al.* 1997). Moreover the oxidative stress resulting due to sustained hyperglycemia is implicated in secondary complications of diabetes. Jovanovic *et al.* (1994) have shown that quercetin has strong radical scavenging potential. It is well evident from the present studies that quercetin combats the oxidative stress produced by vanadium in BQOV leading to reduced ROS generation and better cell survival, as hypothesized.

In vivo vanadium has been shown to cause gastrointestinal disturbances and diarrhea as well as hepatic and renal toxicity. Domingo *et al.* (1991) have demonstrated increased levels of serum urea and creatinine after treatment with vanadium. We have also observed kidney dysfunctionality in $VOSO_4$ injected animals. It is noteworthy that similar to *in vitro* experiments, *in vivo* as well quercetin conjugation has been proved beneficial since there was no oxidative stress in the kidney of BQOV injected animals and serum urea and creatinine levels also remained unaltered. Also quercetin has been shown to protect kidney from ischemia by increasing catalase and SOD activity (Kahraman *et al.* 2003). We have observed an increase in catalase activity in BQOV injected animals.

Amic *et al.* have summarized the radical scavenging activity and structure of flavonoids (2003). In BQOV (Figure 5a), vanadium is conjugated at C3 and C4 positions of quercetin (Figure 5b) and theoretically this does not affect the antioxidant potential of quercetin, which was confirmed practically by estimating SOD IC_{50}. SOD IC_{50} and FRAP value of quercetin and BQOV were found to be comparable indicating retention of radical scavenging activity of the ligand in BQOV. Hence it can be inferred that the reduced ROS found in BQOV could be because of direct scavenging of radicals by the ligand coupled with increased catalase activity.

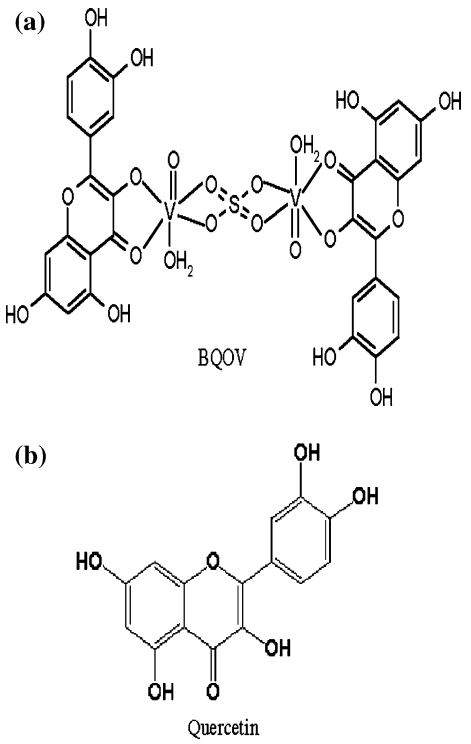

Figure 5. Structure of BQOV (a) and Quercetin (b).

Further it can be speculated that all antioxidants will act as potential organic ligands for vanadium however this does not hold true. Thompson *et al*. (2004) has reported curcumin–vanadium conjugate to be active as an anti-rheumatic, anticancer and anti-arthritic agent but it does not act as an antidiabetic agent. Moreover we have conjugated other flavonoids like morin and 3-hydroxyflavone with vanadium but these conjugates did not exhibit significant hypoglycemic activity (unpublished work). Hence BQOV has a unique blend of high antidiabetic potential and reduced toxicity. Thus present study clearly demonstrates that the quercetin–vanadium conjugate, BQOV, is a viable therapeutic agent, for diabetes and advocates its use as an insulin-mimetic agent.

Acknowledgements

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