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A Comparative Study on the Antioxidant and Glucose-lowering Effects of Curcumin and Bisdemethoxycurcumin Analog through *in vitro* Assays

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Abstract: Type 2 diabetes has emerged as an epidemic affecting millions of people all over the world. Several research findings suggest that oxidative stress is the triggering factor for the development of type 2 diabetes. Hyperglycemia and oxidative stress go hand in hand to accelerate the disease progression in a vicious cycle. In India, the medicinal herb *Curcuma longa* is used as a culinary food additive and also as a therapeutic agent for diabetes. Recent research evidenced that the curcuminoids present in *Curcuma longa* have several medicinal properties including anti-diabetic property. However, the bioavailability of curcumin is very less compared to its derivatives and its synthetic analogs. The synthetic analog of bisdemethoxycurcumin is reported to have increased bioavailability and stability but its effect on controlling oxidative stress, glucose absorption and gluconeogenesis have not yet been explored. The aim of this study was to evaluate the anti-oxidant and glucose lowering effects of bisdemethoxycurcumin analog (BDMCA) in comparison with curcumin. Anti-oxidant activity of BDMCA and curcumin was evaluated by measuring the rate of inhibition of iron-ascorbate induced lipid peroxidation in liver homogenate *in vitro*. Effect of these drugs on intestinal glucose absorption in Wistar rats and on gluconeogenesis in liver homogenate *in vitro* was evaluated to know their glucose lowering effects. We found that both the BDMCA and curcumin lower the gluconeogenesis in the hepatocytes and function as antioxidants *in vitro* in a similar manner. Both BDMCA and curcumin delays the intestinal glucose absorption but BDMCA delays the intestinal glucose absorption more effectively compared to curcumin.

Key words: Curcumin analog, oxidative stress, gluconeogenesis, diabetes, anti-oxidants

INTRODUCTION

The prevalence of type 2 diabetes is rapidly rising all over the globe at an alarming rate and it is correlated with lifestyle changes (Huizinga and Rothman, 2006). Recent research findings suggest that the triggering factor for the disease progression and development of vascular complications is oxidative stress (Kaneto *et al*., 2007). Oxidative stress occurs due to a disturbed balance between pro-oxidants and anti-oxidants in favor of the former. Hyperglycemia is one of the prime factors responsible for the free radical overload in diabetes by enhancing the processes such as auto-oxidation and protein glycation at the same time, inactivating the antioxidant protection. Controlling hyperglycemia and maintaining the antioxidant status in diabetic patients is the corner stone in diabetic management.

Plants have been used as a traditional remedy for numerous diseases for thousands of years in several countries. Turmeric (*Curcuma longa* Linn.) is one such plant, the ground rhizome of which has long been used in the Indian medicine according to the ancient Hindu scripture, the Ayurveda (Brouk, 1975). In addition to its aromatic, stimulant and coloring properties, turmeric has been used for the treatment of wounds, inflammation and tumors (Sharma *et al*., 2007; Chakravarty *et al*., 2009). It is also reported to have anti nociceptive effect (Tajik *et al*., 2007). The rhizome of the *Curcuma longa* contains several curcuminoids such as curcumin, demethoxycurcumin and bisdemethoxycurcumin collectively known as the curcuminoids (Anand *et al*., 2008). The commercial curcumin is a mixture of curcumin, demethoxycurcumin and bisdemethoxycurcumin (Sandur *et al*., 2007).

Although, curcuminoids are having medicinal and pharmacological activities, it is not known how various forms of curcumin differ in their biological action with respect to their structure (Sandur *et al*., 2007). Few reports suggest that bisdemethoxycurcumin is the most potent and stable form of curcumin in the biological systems

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(Fiala *et al*., 2007; Cashman *et al*., 2008; Sandur *et al*., 2007). This compound has been found to possess anti-inflammatory (Guo *et al*., 2008), anti proliferative (Sandur *et al*., 2007) and neuro-protective effects (Fiala *et al*., 2007). Despite having several pharmacological and therapeutic activities, it is not successful clinically due its poor bioavailability, solubility and extensive systemic metabolism (Dhillon *et al*., 2008; Anand *et al*., 2008). Identification of newer curcumin analogues with improved activities is of significant preclinical and clinical interest (Anuradha and Aukunuru, 2010; Simanjuntak *et al*., 2010). Several curcumin derivatives have been synthesized in the laboratory, one such derivative is the bisdemethoxycurcumin analog (BDMCA) (Tomren *et al*., 2007; Babu and Rajasekharan, 1994). Although, natural bisdemethoxycurcumin is reported to have several medicinal properties, the effect of BDMCA on oxidative stress and glucose lowering effect have not been investigated. This study was therefore undertaken to analyze the antioxidant and anti hyperglycemic effects of BDMCA through *in vitro* assays.

MATERIALS AND METHODS

Experimental animals: Male albino rats of Wistar strain aged 9-10 weeks with body weight between 150 to 160 g were procured from the Central Animal House, Rajah Muthiah Medical College, Annamalai University and provided with food and water *ad libitum,* were used in this study. The animals were housed in polypropylene cages under controlled conditions of 12 h light/12 h dark cycle at 24±2°C in a well ventilated room. The experimental protocol followed the guidelines of the National Institute of Nutrition, Indian Council of Medical Research, Hydrabad, India and was approved by the Animal Ethical Committee (Reg. No. 160/1999/CPCSEA), Annamalai University, Tamilnadu, India.

Chemicals: Acetyl acetone, salicyladehyde, N, N-dimethylformamide, boric acid, glacial acetic acid, curcumin, collagenase type IV, HEPES buffer and Thiobarbituric Acid (TBA) were purchased from Sigma-Aldrich, MO, USA. All other chemicals and biochemicals used for the study were of analytical grade.

Preparation of Bisdemethoxycurcumin analog (IUPAC name: bis-1, 7-(4-hydroxyphenyl)-hepta-1, 6-diene-3, 5-dione): BDMCA was prepared according to the previously described method (Babu and Rajasekharan, 1994; Anusuya *et al*., 2003). In brief, acetyl acetone, salicyladehyde, boric acid, N, N-dimethylformamide was refluxed by adding few drops of the catalyst (equal volume of glacial acetic acid and diethanolamine) for 5 to 6 h. The mixture was then poured to 10% acetic acid and stirred for 2 to 3 h. The yellow mass was filtered and the purity was checked by HPTLC (Babu and Rajasekharan, 1994; Anusuya *et al*., 2003). BDMCA is a synthetic analog of natural bisdemethoxycurcumin which differ only in the positioning of hydroxyl group (Babu and Rajasekharan, 1994). The structure of the natural bisdemethoxycurcumin is shown in Fig. 1 and its synthetic analog is shown in the Fig. 2.

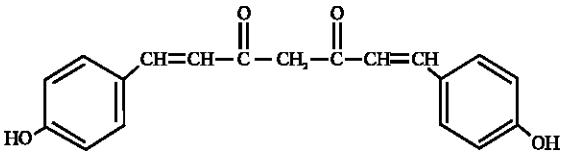

Fig. 1: Structure of natural bisdemethoxycurcumin

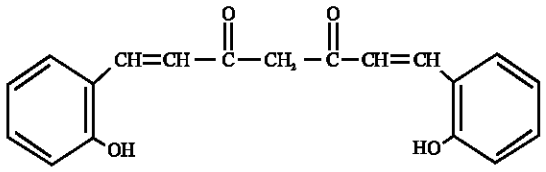

Fig. 2: Structure of natural bisdemethoxycurcumin analog (BDMCA)

HPTLC analysis of BDMCA and curcumin: HPTLC was performed on a pre-activated silica gel coated plate, 20×10 cm of 0.2 mm layer thickness. Equal concentrations of commercial curcumin and BDMCA were dissolved in methanol and applied to the plate as 8 mm wide bands using the Camag sample syringe (Hamilton, Bonduz, Switzerland), using an Automated Camag TLC applicator, Linomat 5 with N2 flow positioned 15 mm from the bottom, 20 mm from side of the plate. The plates were developed in a Camag twin trough glass tank which was pre-saturated with the mobile phase chloroform-methanol (48:2, v/v, 20 mL) for 30 min (Paramasivam *et al*., 2008). The length of the run was 7 cm. The chromatography run was performed under laboratory conditions of 25±5°C and 50% relative humidity. The plates were then air dried. For validation of purity, the TLC spots were photographed at 360 nm using a sony cyber shot digital camera (Fig. 3).

Inhibition of iron-ascorbate induced lipid peroxidation by BDMCA and curcumin: Different concentrations of BDMCA and curcumin were dissolved in DMSO

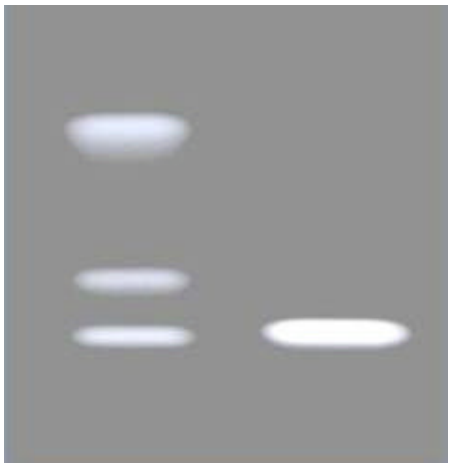

Fig. 3: HPTLC chromatogram detected using digital camera at 360 nm

(Ireson *et al*., 2001) and the ability to subdue iron-ascorbate induced lipid peroxidation was studied in rat liver homogenates (Hogberg *et al*., 1974). The incubation mixture comprised of 0.2 mL of liver homogenate 25% w/v in Tris-HCl buffer, 50 μM $FeSO_4$, 1 mM KH_2PO_4 and 0.2 mM ascorbic acid in 0.15 M Tris-HCl buffer, pH 7.4 and varying concentrations of BDMCA or curcumin were dissolved in DMSO. The mixture was incubated in a shaking water bath at 37°C for 20 min. The reaction was arrested by the addition of 1 mL of 10% trichloroacetic acid. The reaction mixture was shaken well and 1.5 mL of TBA was added and heated in a boiling water bath at 90°C for 20 min. The tubes were centrifuged and the color developed in the supernatant was read at 532 nm. The inhibition of lipid peroxidation was determined by comparing the results of test compound with those of controls not treated with BDMCA or curcumin.

BDMCA and curcumin on glucose absorption: The effect of BDMCA on intestinal glucose absorption was measured. A set of 12 rats maintained on the laboratory diet were starved for 16 h prior to treatment with curcumin or BDMCA at different concentrations (Choudhary *et al*., 1999). Different concentrations of Curcumin or BDMCA dissolved olive oil was given orally. After 10 min, the rats were orally administered glucose (2 g kgG^1 b.wt. dissolved in 0.5 mL water). Blood (about 0.5 mL) was withdrawn from the portal vein under diethyl ether anesthesia at 30 min after the administration of glucose. The concentration of glucose in the blood of portal vein was measured which reflects the amount absorbed in the intestine.

BDMCA and curcumin on gluconeogenesis: Rats were fasted for 24 h prior to the administration of BDMCA or curcumin. BDMCA or curcumin at different concentrations dissolved in olive oil was given by oral gavages (Choudhary *et al*., 1999). Hepatocytes were isolated 24 h after the treatment with BDMCA or curcumin. Pentobarbital (2.5 mg kgG^1) were given subcutaneously to anesthetize the rats. Hepatocytes were isolated. Using the trypan blue exclusion assay, the viability of the isolated hepatocytes was estimated. The cell viability in the control and drug administered rats was greater than 85%. The isolated hepatocytes (5×10^6 cells/2.5 mL) were incubated for 1 h in Krebs-Henseleit buffer (118 mM NaCl, 4.8 mM KCl, 3.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.0 mM KH_2PO_4, 24 mM $NaHCO_3$, 2.5 mM HEPES, pH 7.45) with three different gluconeogenic substrates (20 mM pyruvate, 20 mM lactate, 20 mM alanine). The cells were incubated at 37 °C under an O_2/CO_2 (19:1) atmosphere. After incubation the gluconeogenesis was measured by estimating the glucose content in the culture supernatant after adequate deproteinization and subsequent neutralization. The wet weight of the cells was determined and results are expressed in mmol of glucose produced/min/g wet cells. Statistical analysis was done by Analysis of Variance (ANOVA) followed by Duncan's multiple range test by means of the SPSS version 9.0 for Windows. A value of $p<0.05$ was considered to be statistically significant.

RESULTS

The purity of the synthesized BDMCA was assayed by HPTLC and it was compared with commercial curcumin. The commercial curcumin gave rise to three bands such as curcumin, demethoxycurcumin and bisdemethoxycurcumin, as it was reported early (Paramasivam *et al*., 2008), while the synthetic BDMCA gave rise to only one band showing its homogenous nature (Fig. 3). Table 1 shows the inhibitory effect of BDMCA and natural curcumin on iron-ascorbate induced lipid peroxidation *in vitro*. BDMCA significantly reduced (0.17±0.014 and 0.08±0.008) the iron-ascorbate induced lipid peroxidation in a dose-dependent manner when compared to control sample (0.29±0.022) as shown by the amount of TBARS produced. Curcumin also showed a dose-dependent inhibition (0.16±0.008 and 0.07±0.081) on the iron-ascorbate induced lipid peroxidation *in vitro*. BDMCA and curcumin have similar effect at similar dose in the iron-ascorbate induced lipid peroxidation.

Table 2 shows the effect of BDMCA and curcumin on intestinal glucose absorption in Wistar rats. Oral

Table 1: Inhibition of iron-ascorbate induced lipid peroxidation by BDMCA and curcumin

Groups	TBARS (nmoles mg^{-1} protein)
Liver homogenate treated with iron -ascorbate	0.29±0.022[a]
Liver homogenate treated with iron-ascorbate plus BDMCA 25 (ng mL^{-1})	0.17±0.014[b]
Liver homogenate treated with iron-ascorbate plus BDMCA 50 (ng mL^{-1})	0.08±0.008[c]
Liver homogenate treated with iron-ascorbate plus curcumin 25 (ng mL^{-1})	0.16±0.008[b]
Liver homogenate treated with iron-ascorbate plus curcumin 50 (ng mL^{-1})	0.07±0.081[c]

Results are Mean±SD of five measurements. Values not sharing common superscript vary significantly each other. $p<0.05$, ANOVA followed by DMRT

Table 2: Inhibitory effect of BDMCA and curcumin on intestinal glucose absorption

Groups	Glucose levels in portal vein (mM)
Control rats	6.60±0.50[a]
Control rats treated with vehicle alone	6.30±0.47[a]
Rats treated with only glucose	18.68±1.42[e]
Rats treated with BDMCA (10 mg kg^{-1}) and glucose	9.54±0.72[b]
Rats treated with BDMCA (20 mg kg^{-1}) and glucose	7.23±0.55[a]
Rats treated with Curcumin (10 mg kg^{-1}) and glucose	14.59±1.11[d]
Rats treated with Curcumin (20 mg kg^{-1}) and glucose	11.34±0.86[c]

Results are Mean±SD of five measurements. Values not sharing common superscript vary significantly each other. $p<0.05$, ANOVA followed by DMRT

Table 3: Effect of BDMCA and curcumin on gluconeogenesis

Groups	Pyruvate (mmol/min/g wet cells)	Lactate (mmol/min/g wet cells)	Alanine (mmol/min/g wet cells)
Control	0.42±0.03[a]	0.34±0.02[a]	0.44±0.03[a]
BDMCA 25 ng mL^{-1}	0.31±0.22[b]	0.23±0.01[b]	0.25±0.01[b]
BDMCA 50 ng mL^{-1}	0.26±0.01[c]	0.19±0.01[c]	0.18±0.01[c]
Curcumin 25 ng mL^{-1}	0.32±0.02[b]	0.25±0.01[b]	0.23±0.01[b]
Curcumin 50 ng mL^{-1}	0.25±0.01[c]	0.19±0.01[c]	0.17±0.01[c]

Results are Mean±SD of five measurements. Values not sharing common superscript vary significantly each other. $p<0.05$, ANOVA followed by DMRT

administration of BDMCA significantly decreased (9.54±0.72 and 7.23±0.55) the intestinal glucose absorption in a dose-dependent manner when compared to control (6.6±0.50) rats. Curcumin treatment also decreased the intestinal glucose absorption in a dose dependent manner (14.59±1.11 and 11.34±0.86), but it is less significant when compared to BDMCA (9.54±0.72 and 7.23±0.55).

Table 3 shows the effect of pretreatment with BDMCA and curcumin on gluconeogenesis in liver homogenate of Wistar rats. Pretreatment with BDMCA or curcumin significantly decreased the hepatic gluconeogenesis in the liver homogenate (provided with different gluconeogenic substrates) when compared with control (Table 3). There was no significant variation between BDMCA and curcumin in decreasing gluconeogenesis at the corresponding similar doses.

DISCUSSION

Curcuminoids has been shown to exhibit anti-inflammatory, antimicrobial and anti-carcinogenic activities (Anand *et al*., 2008). Despite having wide spectrum pharmacological activities, the utility of curcuminoids is limited by its color, lack of water solubility and relatively low *in vivo* bioavailability (Anand *et al*., 2008). So there is an intense search for a natural or synthetic super curcumin without these limitations (Anand *et al*., 2008). Several curcumin analogs are being synthesized in the laboratory by different researchers (Youssef and El-Sherbeny, 2005; Mosley *et al*., 2007; Lin *et al*., 2006). BDMCA is one such synthetic analog of natural bisdemethoxycurcumin which differ only in the positioning of hydroxyl group (Babu and Rajasekharan, 1994). We have synthesized BDMCA is our laboratory and the purity was assessed by HPTLC in comparison with commercial curcumin (Fig. 1 and 2). We found that the synthesized BDMCA was homogenous as shown by the HPTLC bands while the commercial curcumin is comprised of curcumin, demethoxycurcumin and bisdemethoxycurcumin and it was separated into to three bands (Paramasivam *et al*., 2008) (Fig. 3).

We have studied the antioxidant activity of BDMCA by measuring its ability to inhibit the iron-ascorbate induced lipid peroxidation by measuring the lipid peroxidation product TBARS (Hogberg *et al*., 1974). From the results, it was observed that BDMCA inhibits the iron-ascorbate induced lipid peroxidation in liver homogenate dose dependently. We have compared the antioxidant effect of BDMCA with natural curcumin. We found that at the similar dose, the inhibitory effect of BDMCA and the commercial curcumin is same.

It has been reported previously that the natural curcuminoids such as curcumin, demethoxycurcumin and bisdemethoxycurcumin are equipotent in suppressing iron-induced lipid peroxidation (Sreejayan-Rao, 1994). In this study we have found that the synthetic analog BDMCA which differ only in the positioning of hydroxyl group possess antioxidant activity similar to natural curcumin. The antioxidant activity of synthetic BDMCA might be due to its phenolic group and the central methylenic hydrogen in the central seven-carbon chain and b-diketone moiety (Sandur *et al*., 2007). Previous reports have suggested that the donation of hydrogen atom from the beta-diketone moiety to free radical such as the lipid alkyl or peroxyl radical is responsible for its antioxidant activity (Jovanovic *et al*., 2001). As the beta-diketone moiety donates a hydrogen atom, the curcumin became a curcumin radical with unpaired electron density between the two oxygen atoms. The main

radical that is formed is the phenoxyl radical (Jovanovic *et al*., 2001). Curcumin is a potent lipid-soluble antioxidant and penetrates deep into the cell membrane where it scavenges free radicals and thereby converts itself into the phenoxyl radical (Jovanovic *et al*., 2001). Since, this radical is more polar than curcumin, it can move to the membrane's surface where it could be repaired by water soluble antioxidants which could present in the homogenate.

Management of diabetes involves the delaying the intestinal absorption of glucose so as to minimize the post-prandial hyperglycemia in the diabetic patients. We have analyzed the effect of BDMCA and curcumin on intestinal glucose absorption in the Wistar rats. From the results we have found that BDMCA inhibited the intestinal glucose absorption more effectively than natural curcumin. It has been reported previously that turmeric posses hypoglycemic effect (Tank *et al*., 1990). GLUT2 is involved in the intestinal transport of glucose. Curcumin is a polyphenolic compound, with structural similarities to phloretin. Furthermore, phloretin is known to inhibit GLUT2 and has recently been shown to contribute significantly to the uptake of glucose from the small intestine (Kellett and Brot-Laroche, 2005). BDMCA might have delayed the glucose absorption by a similar mechanism to that of curcumin. It is not known exactly how BDMCA is more potent in delaying the intestinal glucose absorption when compared to curcumin. As BDMCA structurally differs from the natural curcuminoids (Babu and Rajasekharan, 1994), it might have differently interacted with the glucose transporter thereby delayed the intestinal glucose absorption more potently than curcumin. Moreover, the effects of BDMCA in the intestine are suggestive of increased bioavailability of BDMCA than curcumin.

Gluconeogenesis primarily takes place in the liver which plays a unique role in regulating carbohydrate metabolism to maintain a normal glucose concentration in the blood (Saltiel, 2001). BDMCA and curcumin reduced gluconeogenesis in the presence of gluconeogenic substrates in the rat hepatocytes compared to control. The inhibitory effect was comparatively similar in the BDMCA and curcumin-treated samples. Previously it has been reported that curcumin lowers blood glucose in experimental animals (Arun and Nalini, 2002; Majithiya and Balaraman, 2005; Weisberg *et al*., 2008) and inhibit the gluconeogenic enzymes such as glucose-6-phosphatase and phosphoenol pyruvate kinase (Seo *et al*., 2008). The inhibitory effect of BDMCA and curcumin on gluconeogenic enzymes might be attributed to the down-regulation of the gluconeogenesis *in vitro*.

CONCLUSION

From the study it can be concluded that BDMCA acts as an anti-oxidant *in vitro*, decreases the intestinal glucose absorption and also inhibits the gluconeogenesis *in vitro*. The effects are comparable to curcumin. However BDMCA is more potent in delaying intestinal glucose absorption than curcumin. Further studies using *in vivo* animal models could reveal more information about BDMCA for its use as an anti-oxidant and glucose lowering agent.

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