Effect of natural products on commercial oral antidiabetic drugs in enhancing 2-deoxyglucose uptake by 3T3-L1 adipocytes

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***Abstract*:**
Objective: The management of diabetes with insulin and synthetic oral hypoglycemic drugs (OHDs) can produce serious side effects and in addition fails to prevent diabetes-related complications in many patients. A new diabetes management strategy is needed that is more effective and has fewer side effects.
Methods: This paper analyzes the dose- and time-dependent effect of three phytochemicals: berberine, arecoline and vanillic acid, and two antidiabetic drugs: 2,4-thiazolidinedione (TZD) and metformin, on the uptake of 2-deoxyglucose (2DG) by 3T3-L1 adipocytes. The interactions of the phytochemicals with the OHDs were analyzed with isobolograms and the combination index.
Results: TZD and berberine increased 2DG uptake by 3.3-fold (with respect to control) at 15 μM and 25 μM, respectively. The same concentrations of arecoline and vanillic acid increased 2DG uptake by 3.2-and 2.9-fold, respectively, when compared with the basal level. Berberine and arecoline acted synergistically with both the OHDs, whereas vanillic acid had an additive interaction with TZD and an antagonistic interaction with metformin. Arecoline significantly increased the translocation of GLUT4 via the PPARγ pathway, whereas berberine and vanillic acid did this via the AMPK-dependent pathway.
Conclusions: These phytochemicals significantly reduced the expression of the enzymes involved in fatty acid and cholesterol synthesis, indicating that they might help prevent the secondary complications of diabetes. The current study suggests that berberine and arecoline could allow dosage reduction of OHDs, which could also lead to a reduction in the toxicity and side effects caused by OHDs.

***Keywords*:** berberine, 2-deoxyglucose, isobologram, synergy, vanillic acid

Introduction

Diabetes mellitus (DM) is a chronic endocrine metabolic disorder characterized by hyperglycemia. It is caused by inadequate insulin, either in its production or its function [Harris and Zimmet, 1997]. The disorder can be classified into type 1 (insulin-dependent DM) and type 2 (noninsulin-dependent DM). The latter accounts for more than 90% of all diabetes cases and is caused mainly by peripheral insulin resistance and impaired insulin secretion. It is often associated with lipid and lipoprotein disorders [Ciresi *et al.* 2007; Oh *et al.* 2005]. The metabolic disorder includes alterations in carbohydrates, lipids and protein metabolism.

Management of diabetes with insulin and synthetic oral hypoglycemic drugs (OHDs) can produce serious side effects and fails to prevent diabetes-related complications in many patients. Herbal remedies are effective, produce minimal or no side effects in clinical experience and are of relatively low cost compared with synthetic OHDs [Momin, 1987]. More than 800 plants are reported to be traditional remedies for the treatment of diabetes [Prabhakar and Doble, 2008a, 2008b] and many Indian medicinal plants have been reported to manage diabetes. The search for more effective and safer hypoglycemic agents has continued to be an important area of active research and recommendations

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made by World Health Organization have speeded up investigations on hypoglycemic agents from medicinal plants.

Detailed pharmacological experiments with single isolated compounds *versus* original extract or extract fractions have confirmed that many plant constituents, among them primarily phenolic compounds and terpenoids, exert polyvalent pharmacological effects [Wagner and Ulrich-Merzenich, 2009]. Many phytochemicals and pharmaceuticals are therapeutic at one dose and toxic at another and synergistic interactions can complicate dosing during long-term medication but experimental data on herb–drug interactions are limited [Fugh-Berman, 2000]. Reports indicate that the interaction of garlic with chlorpropamide [Aslam *et al.* 1979], gingko with tolbutamide [Uchida *et al.* 2006] and St John′s wort with gliclazide and tolbutamide [Xu *et al.* 2008; Arold *et al.* 2005] do not change the pharmacokinetics of the pharmaceutical. Administration of antidiabetic herbs with OHDs for the treatment of diabetes could cause a drug–herb interaction that might have beneficial or adverse effects. It is generally believed that the use of herbs with pharmaceuticals enhances the effect of the latter and reduces their adverse effects [Williamson, 2001].

This paper describes the effect of three phytochemicals: berberine, arecoline and vanillic acid alone and in combination with two commercial OHDs: metformin and 2,4-thiazolidinedione (TZD) on the transport of 2-deoxyglucose (2DG) in 3T3-L1 adipocytes. Uptake was measured with a diaphorase–resazurin system that produces a fluorescent substance in the presence of NADPH, which is measured with a fluorescence spectroscope. 2DG was more convenient to use than glucose because it is phosphorylated by hexokinase or glucokinase to a stable and impermeable derivative, 2-deoxyglucose-6-phosphate (2DG6P), and we used a nonradioisotopic assay [Yamamoto *et al.* 2006].

Berberine, an isoquinoline alkaloid, is a principal component of the roots, rhizome and bark of several plants, including *Berberis aristata* L. and *Coptis chinensis* Franch. (coptis root; rhizoma coptidis). These plants have been used for the treatment of diarrhea and other gastrointestinal disease in China and other Asian countries. In traditional Chinese medicine, berberine is also used for weight reduction. Recent studies have shown that berberine exhibits multiple pharmacological activities against cardiovascular diseases, inflammation and hypercholesterolemia [Birdsall and Kelly, 1997]. It is generally regarded as safe in recommended dose. However, higher doses (>0.5 g) may cause lethargy, dizziness, dyspnea and skin and eye irritation [Prabhakar and Doble, 2009]. There is little information about its metabolic fate in animals or humans; however three different berberine metabolites have been observed in human urine after oral administration [Jun-Fang *et al.* 2002].

Arecoline is an alkaloid found in the fruits of the areca palm (*Areca catechu* L.). It is known to be a partial agonist of muscarinic acetylcholine M_1, M_2 and M_3 receptors. It is extensively metabolized to multiple products; none via cytochrome P450. The metabolism involves mainly *N*-oxidation reactions [Giri *et al.* 2006].

Vanillic acid (4-hydroxy-3-methoxybenzoic acid) is a natural plant phenolic compound from vanilla and is used in traditional Chinese medicine. Treatment of Neuro-2A cells with phenolic acids markedly suppresses cell apoptosis induced by methylglyoxal, suggesting that phenolic acids may possess cytoprotective ability and help prevent the peripheral neuropathy complications of diabetes [Huang *et al.* 2008].

Materials and methods

The 3T3-L1 adipocyte cell line, derived from mouse muscle fibroblasts, was purchased from NCCS, Pune, India. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin and antibiotics (penicillin and streptomycin) were purchased from PAN BIOTECH GmbH, Germany. 2DG, hexokinase, glucose-6-phosphate dehydrogenase (G6PDH), diaphorase, resazurin, ATP, $NADP^+$ and all the primers were obtained from Sigma Aldrich, Bangalore, India. TZD and bovine serum albumin (BSA) were purchased from Himedia Laboratories Pvt Ltd, Mumbai, India and metformin and dimethyl sulfoxide (DMSO) were purchased from Merck, Mumbai, India. All other chemicals were procured from SRL Mumbai, India. The Medox easy spin column total RNA Miniprep super kit was purchased from Medox Biotech, Chennai, India, and was used for the total RNA extraction. RobusT I RT-PCR kit was purchased from Finzymes, Finland. All the primers were purchased from Bioserve, Hyderabad, India and plastic wares from Tarson, Kolkata, India.

Cell culture of 3T3-L1 adipocytes

3T3-L1 cells were grown and maintained in DMEM with 100 U/ml penicillin, 100 µg/ml streptomycin, and 1 mM sodium pyruvate, supplemented with 10% FBS. Cells were grown at 37°C in 5% CO_2 in a humidified chamber and reefed with the fresh medium every 2 days. For differentiating the adipocytes, cells were grown in 24-well plates to full confluence for 2 days and the differentiation medium, containing 10 µg/ml insulin and 0.5 μM dexamethasone, was added to the culture. After 4 days of induction the medium was changed to DMEM with 10% FBS and maintained at 37°C and 5% CO_2.

Determination of 2-deoxyglucose

We estimated 2DG in a 24-well plate using an enzymatic diaphorase–NADPH amplifying system [Yamamoto *et al.* 2006]. We dispensed 100 μl of 2DG solutions of different concentrations (0.25, 0.5, 1, 2, 5 and 10 μM) into each well and incubated for 90 min at 37°C after the addition of 300 μl of a reaction cocktail consisting of 50 mM TEA (Triethanolamine) Buffer (pH 8.1), 0.02% BSA, 50 mM KCl, 0.5 mM $MgCl_2$, 670 μM ATP, 0.12 μM $NADP^+$, 25 μM resazurin sodium salt, 5.5 U/ml hexokinase, 16 U/ml G6PDH, and 1 U/ml diaphorase. This cocktail was prepared from the stock solutions just before the assay and refrigerated. After 90 min of incubation, the fluorescence at 590 nm with excitation at 530 nm was measured with an FP-6500 research grade fluorescence spectrometer (JASCO International Ltd, Japan), to detect the conversion of resazurin to resorufin. The amount of resorufin formed is theoretically equivalent to the amount of 2DG uptake if the reaction goes to completion.

2-deoxyglucose uptake

The differentiated adipocytes were starved of serum for 4 h in DMEM then twice rinsed with Krebs–Ringer phosphate HEPES (KRPH) buffer (pH 7.4, 20 mM HEPES, 5 mM KH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 136 mM NaCl, 4.7 mM KCl). The cells were incubated with 350 μl/well DMEM and 2% FBS in the presence of the test compounds (metformin, 2,4-thiazolidinedione, arecoline, vanillic acid or berberine) for a specific time. After incubation the cells were washed twice with KRPH buffer containing 0.1% BSA then incubated with KRPH buffer containing 1.5 mM 2DG and 0.1% BSA for 30 min at 37°C in 5% CO_2. Later, they were rinsed with KRPH buffer containing 0.1% BSA and 50 μl of NaOH (0.1N). The alkalinity in the wells was neutralized by the addition of 50 μl HCl (0.1N), followed by 50 μl of 150 mM TEA buffer (pH 8.1). The fluorescence was measured by the enzymatic method described above, which was an indication of the concentration of 2DG inside the cells.

For dose-dependent studies nine different concentrations (0, 2.5, 5, 7.5, 10, 15, 20, 25 and 50 µM) of the compounds were selected, and for time-dependent studies five different time points (between 1 and 5 h with a 1 hour gap) were chosen. In the combination study five different concentrations of the OHDs (2.5, 5, 10, 15, 20 and 25 µM) and six different concentrations (2.5, 5, 10, 15, 20 and 25 µM) of the phytochemicals were tested. All the experiments were performed in triplicate and the mean ± standard deviation (SD) reported.

The interaction of a phytochemical with a commercial OHD was ascertained using a calculated parameter termed the combination index (CI) [Zhao *et al.* 2005], calculated by

$$\text{Combination Index (CI)} = \left[\frac{C_a}{IC_a}\right] + \left[\frac{C_b}{IC_b}\right]$$

where C_a and C_b are the concentrations of compound A and compound B used in combination to achieve a fixed effect (in this case a certain amount of glucose uptake). IC_a and IC_b are the concentrations of the two compounds required to achieve the same effect when used alone. A CI value less than, equal to, or more than 1 indicates synergistic, additive and antagonistic interaction, respectively, between the two compounds.

The effect of two compounds on 2DG uptake is also shown in the form of an isobologram. The *x*- and *y*-axes of the isobologram are the concentrations of the compounds A and B required individually and in combination to achieve the same amount of 2DG uptake by the cells. The straight line connecting the individual concentrations of the compound (when used alone) required to achieve the same level of 2DG uptake is the 'line of additivity'. If the combination curve is well below this straight line (based on 95% confidence limits) then the interaction is deemed synergistic and if it is above the line then it is antagonistic [Prabhakar and Doble, 2010].

Reverse transcription polymerase chain reaction

The modulation of the genes involved in the insulin cascade (*GLUT4*, *PPARγ*, *PI3K* and *AMPK*), the adipogenic enzymes (fatty acid synthase and HMG-CoA reductase) and the proinflammatory cytokines tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) was determined using reverse transcription polymerase chain reaction (RT-PCR) as described in the literature [Hall *et al.* 1998]. The total RNA from 3T3-L1 adipocytes was isolated after overnight incubation with the phytochemical or the commercial OHDs using a total RNA Miniprep super kit as per the manufacturer's instructions. RT-PCR was carried out with 5 U/μl of avian myeloblastosis virus reverse transcriptase along with 20 pg of template RNA to obtain the cDNA. The primers used are listed in Table 1. The PCR reaction mix consisted of 10 × PCR buffer, 10 mM of each of the dNTP, 10 pmol of paired primers, two units of DNA polymerase and distilled water, to make a total volume of 50 ml. The mixture was overlaid with mineral oil and subjected to 30 cyclic reactions in a PTC-200 DNA Engine® PCR thermal cycler (MJ Research, CA, USA). The products were run on 1.5% agarose gel, stained with ethidium bromide and photographed. The intensity of the bands was measured with GelDock® (Biorad, USA). The density of the band for the control sample (without any compound) was considered as one and the density of the bands obtained with OHDs or phytochemicals were expressed in multiples of this value.

Calculation of absorption, distribution, metabolism and excretion properties

The physiological process involved in the absorption, distribution, metabolism and excretion (ADME) of a compound is an important determinant of its therapeutic efficacy. There are many commercial software packages that can theoretically estimate these parameters for a given chemical structure. The structures of the three phytochemicals and the two OHDs were drawn using Hyperchem® software (HyperCube Inc., USA) and their minimum energy conformations were obtained using the MM + force field. QikProp® (Schrödinger Inc., Portland, USA) software was used for calculating the ADME and the drug-likeness properties of these compounds. The descriptors for these properties are QPlogBB (predicted brain/blood partition coefficient), percent human oral absorption (predicted human oral absorption on a 0–100% scale, based on a quantitative multiple linear regression model), QPlogS (predicted aqueous solubility), number of violations of Lipinski's rule of five (the rule is: molecular weight <500, log o/w partition coefficient <5, number of hydrogen bond donor ≤5, number of hydrogen bond acceptor ≤10) and number of violations of Jorgensen's rule of three (the rule is: predicted aqueous solubility (QPlogS)> −5.7, predicted apparent Caco-2 cell permeability >22 nm/s, number of primary metabolites <7) [Dossi *et al.* 2009; Duchowicz *et al.* 2007]. The recommended ranges are: blood–brain partition coefficient −3.0 to 1.2, percent human oral absorption 25 to 80%, QPlogS −6.5 to 0.5, and the permitted violations of the rule of five and rule of three are 1 and 0, respectively.

Statistical analysis

All of the experiments were performed in triplicate and the results expressed as mean ± SD. Student's t-test was performed on the data by using Systat® software (Systat, Chicago, USA). A p value <0.05 was considered as significant.

Results and discussion

Toxicity of 2-deoxyglucose to 3T3-L1 adipocytes

The MTT assay (method supplied in the online Supplementary Material), which measures the activity of the mitochondrial reductase enzyme, is an estimate of the number of viable cells [Mosmann, 1983]. When treated with the test compounds at a concentration of 50 μM for 24 h, 80–85% of the 3T3-L1 adipocytes were viable compared with control (Supplementary Figure 1). None of the compounds was cytotoxic at concentrations below 50 μM but they became toxic above 100 μM. No visible changes in cell morphology or attachment were observed as a result of these treatments. No statistically significant differences were observed in the toxicity induced by these compounds at a concentration of 50 μM.

Differentiation assay (triglyceride content)

Triglyceride (TG) was estimated with the Oil Red O assay (method supplied in the online Supplementary Material) [Inazawa *et al.* 2003]. The phytochemicals (at 50 μM) increased the differentiation of 3T3-L1 adipocytes less than control (treated with differentiation media) or TZD (Figure 1). When adipocytes were treated with TZD and metformin the TG contents were 84% and 67%, respectively ($p < 0.01$), with

Table 1. Primers used for reverse transcription polymerase chain reaction.

Gene	Sequence	Orientation
GLUT4	5′-CCAGCCTACGCCACCATAG-3′	Forward
	5′-TTCCAGCAGCAGCAGAGC-3′	Reverse
PPARγ	5′-AGGGCCCTGTCTGCTCTGTG-3′	Forward
	5′-TACCAGCTTGAGCAGCACAAGTCG-3′	Reverse
PI3K	5′-TGA CGC TTT CAA ACG CTA TC-3′;	Forward
	5′-CAG AGA GTA CTC TTG CAT TC-3′	Reverse
TNF-α	5′-ACC TTT CCA GAT TCT TCC CTG AG-3′	Forward
	5′-CCC GGC CTT CCA AAT AAA TAC ATT-3′	Reverse
IL-6	5′-GAG GAT ACC ACT CCC AAC AGA CC-3′	Forward
	5′-AAG TGC ATC ATC GTT GTT CAT ACA-3′	Reverse
HMG-CoA reductase	5′-CAT GCA GAT TCT GGC AGT CAG T-3′	Forward
	5′-CGG CTT CAC AAA CCA CAG TCT-3′	Reverse
Fatty acid synthase	5′-CTG CGT GGC TAT GAT TAT GGC-3′	Forward
	5′-CGT GAG GTT GCT GTC GTC TGT-3′,	Reverse

IL-6, interlerleukin 6; TNF-α, tumor necrosis factor alpha.

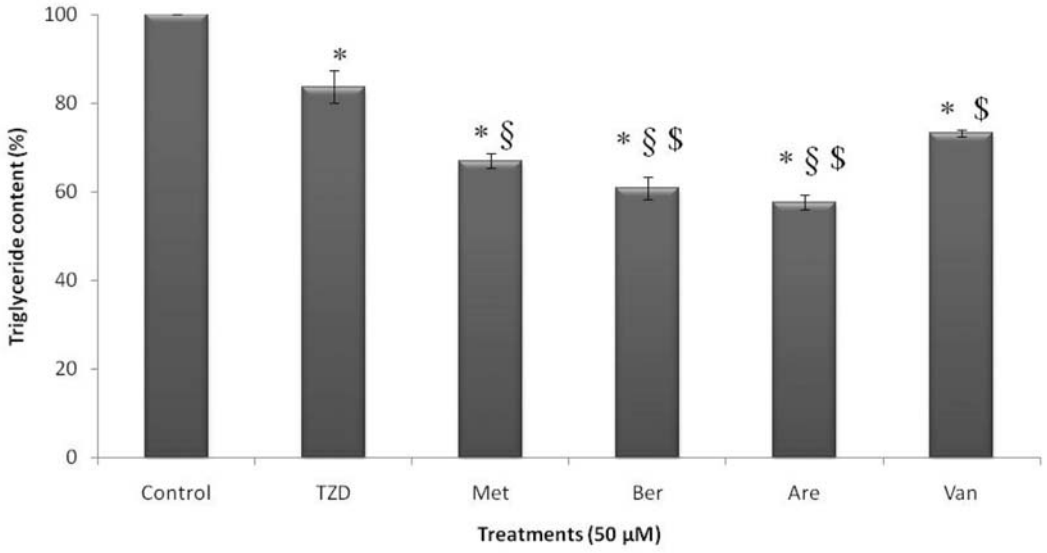

Figure 1. Effect of various treatments for 4 h on triglyceride levels in 3T3-L1 adipocytes. *$p < 0.05$ when compared with control; §$p < 0.05$ when compared with 2,4-thiazolidinedione; $$p < 0.05$ when compared with metformin. Are, arecoline; Ber, berberine; Met, metformin; TZD, 2,4-thiazolidinedione; Van, vanillic acid.

reference to the control. The TG concentrations inside the differentiated adipocytes treated with phytochemicals were less than the cells treated with TZD but comparable with the cells treated with metformin. The accumulated TG in the case of vanillic acid-treated cells was 73% of the control and was more than in berberine- and arecoline-treated cells (61% and 56%, respectively).

Dose-dependent and time-dependent glucose uptake

The phytochemicals and OHDs increased 2DG uptake by 3T3-L1 adipocytes in a dose- and time-dependent manner (Figure 2). In all the cases the maximum 2DG uptake was reached between 3 and 4 h of incubation (Figure 2a). TZD at a concentration of 15 µM induced glucose uptake by 3.3-fold above the basal level, whereas berberine induced 2DG uptake by 3.3-fold at a concentration of 25 µM. The same concentration of arecoline and vanillic acid increased 2DG uptake by 3.2- and 2.9-fold, respectively, when compared with the basal level (Figure 2b).

Interaction of phytochemicals with commercial antidiabetic drugs

The interactions between OHDs and phytochemicals were analyzed with isobolograms and

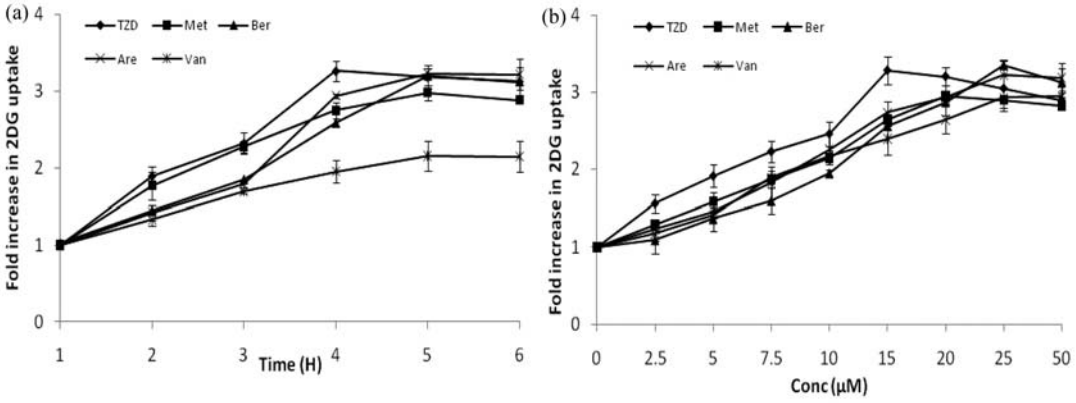

Figure 2. (a) Time-dependent and (b) dose-dependent 2-deoxyglucose uptake by 3T3-L1 adipocytes in the presence of oral hypoglycemic drugs and phytochemicals. Are, arecoline; Ber, berberine; Met, metformin; TZD, 2,4-thiazolidinedione; Van, vanillic acid.

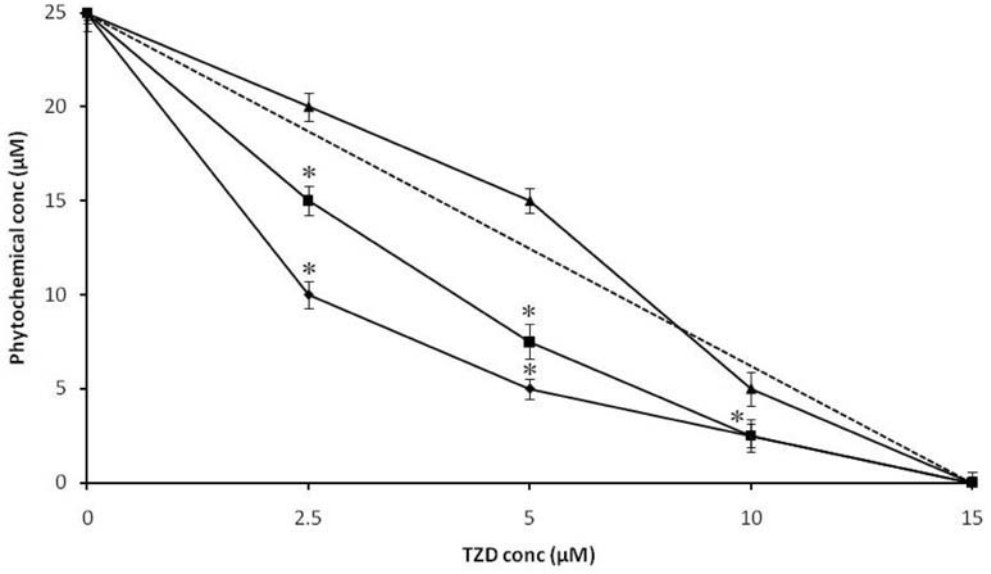

Figure 3. Isobologram depicting the effect of the interaction of phytochemicals with 2,4-thiazolidinedione (TZD) on 2-deoxyglucose (2DG) uptake of 263 ng/3.5×10^5 3T3-L1 adipocytes (berberine ◆, arecoline ■, vanillic acid ▲; *$p < 0.05$ when compared with control). Dotted line indicates line of additivity. Berberine and arecoline act in synergy with TZD on the uptake of 2DG.

the CI. The isobolograms relating the concentrations of various phytochemicals in combination with TZD to achieve a 2DG uptake of 263 ng/3.5×10^5 3T3-L1 adipocytes (i.e. a 3.29-fold increase in 2DG uptake, which is the maximum uptake with TZD) is shown in Figure 3. Similarly, the isobolograms relating the concentrations of various phytochemicals in combination with metformin to achieve a 2DG uptake of 236 ng/3.5×10^5 3T3-L1 adipocytes (i.e. 2.95-fold increase in 2DG uptake, which is the maximum uptake with metformin) is shown in Figure 4. Berberine and arecoline showed synergistic interaction with both the OHDs (the combination curve is below the line of additivity), whereas the curves for vanillic acid with both the OHDs were close to or above the line, indicating additive or antagonistic behavior, respectively.

TZD (20 μM) in combination with berberine (25 μM), arecoline (25 μM) or vanillic acid (50 μM) increased 2DG uptake by 6.4−, 6.2− and 5.84-fold, respectively, compared with

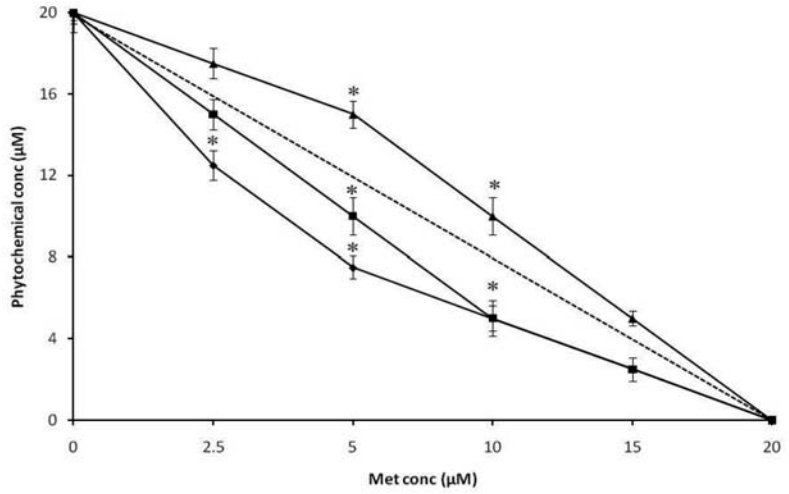

Figure 4. Isobologram depicting the interaction of phytochemicals with metformin for a 2-deoxyglucose (2DG) uptake of 237 ng by 3.5×10^5 3T3-L1 adipocytes (berberine ◆, arecoline ■, vanillic acid ▲; $*p < 0.05$ when compared with control). Dotted line indicates line of additivity. Berberine and arecoline act in synergy with 2,4-thiazolidinedione in the uptake of 2DG; vanillic acid exhibits antagonistic behavior.

control. Metformin (20 μM) in combination with berberine (25 μM), arecoline (25 μM) or vanillic acid (50 μM) increased 2DG uptake by 6.1−, 5.97− and 5.3−fold, respectively, when compared with control.

The CI for vanillic acid with TZD at various ratios was almost equal to 1, indicating additive behavior, whereas the CIs for the other two phytochemicals with TZD at different ratios were less than 1, indicating synergy (Supplementary Tables S1, S3 and S5). Similar observations were made for the interaction between metformin and the phytochemicals (Supplementary Tables S2, S4 and S6).

Berberine 10 μM or arecoline 15 μM reduced by one-sixth the dose of TZD required to achieve a 3.3-fold increase in 2DG uptake. Similarly, 7.5 μM berberine or 10 μM arecoline reduced by one-quarter the amount of metformin required for the same 2DG uptake.

Reverse transcription polymerase chain reaction

The mechanism of action of the three phytochemicals and the two OHDs were investigated by monitoring the modulation of the *GLUT4*, *PPARγ*, *PI3K* and *AMPK* genes at transcript level by semiquantitative RT-PCR. A significantly elevated expression of GLUT4 (Figure 5) was observed following treatment with phytochemicals and OHDs. Arecoline increased the expression of *PPARγ*, whereas berberine and vanillic acid significantly increased the expression of *AMPK*. No significant effect on the expression of *PI3K* was observed for any of the phytochemicals.

Densitometric scanning of the gel is shown in Figure 6. Berberine, arecoline, vanillic acid, TZD and metformin increased the expression of *GLUT4* by 2.73−, 2.71−, 1.42−, 3.35− and 2.65−fold, respectively when compared with control. They also increased the expression of *PPARγ* by 1.57−, 2.3−, 1.8−, 2.73− and 1.52−fold, respectively and *AMPK* by 2.73−, 1.47−, 1.85−, 1.58− and 3.1−fold, respectively. These results indicate that arecoline increases glucose uptake via the *PPARγ* pathway, whereas berberine and vanillic acid increase glucose uptake via the *AMPK* pathway.

These phytochemicals significantly decreased the expression of fatty acid synthase (involved in fatty acid synthesis) and HMG-CoA reductase (involved in cholesterol synthesis) (Figure 7). This suggests that these phytochemicals may reduce the secondary complications observed during diabetes. Densitometric scanning (Figure 8) shows the reduction in the expression of fatty acid synthase and HMG-CoA reductase by berberine (63 and 72%, respectively), arecoline (51 and 57%, respectively), vanillic acid (39 and 46%, respectively), metformin (22 and 39%, respectively) and TZD (35 and 24%,

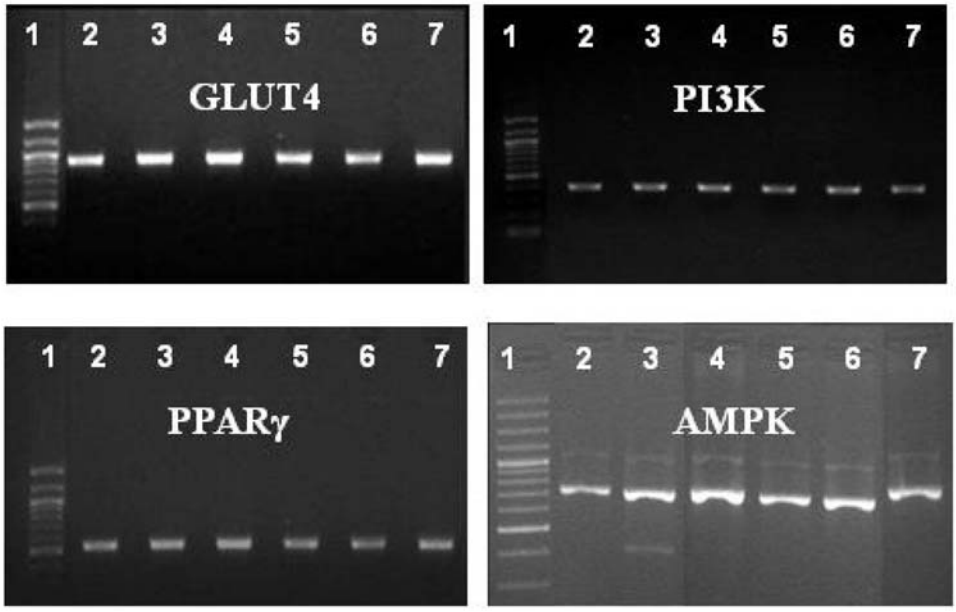

Figure 5. The effect of phytochemicals and oral hypoglycemic drugs on the expression of *GLUT4*, *PPARγ*, *PI3K* and *AMPK* transcripts in 3T3-L1 adipocytes, as determined by reverse transcription polymerase chain reaction. Lane 1 = 100 bp marker, lane 2 = control (no treatment), lane 3 = 2,4-thiazolidinedione, lane 4 = metformin, lane 5 = berberine, lane 6 = arecoline, lane 7 = vanillic acid.

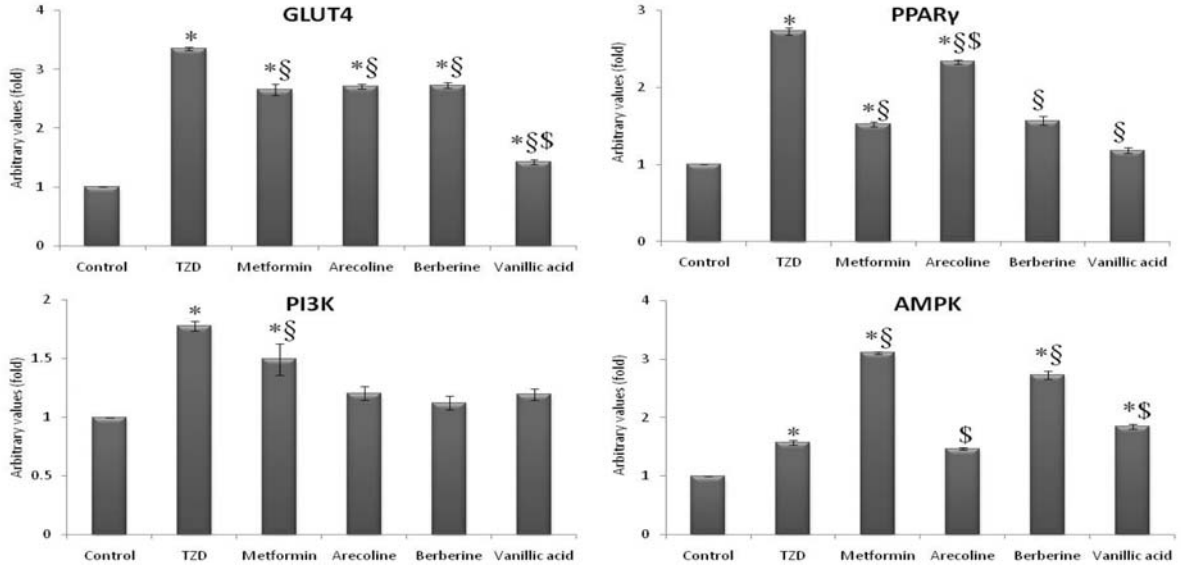

Figure 6. Densitometric scanning of *GLUT4*, *PPARγ*, *PI3K* and *AMPK* transcripts in the presence of commercial drugs and natural products. Bars represent mean ± SD of three independent experiments. *$p < 0.05$ when compared with control; §$p < 0.05$ when compared with 2,4-thiazolidinedione; \$$p < 0.05$ when compared with metformin. Are, arecoline; Ber, berberine; Met, metformin; TZD, 2,4-thiazolidinedione; Van, vanillic acid.

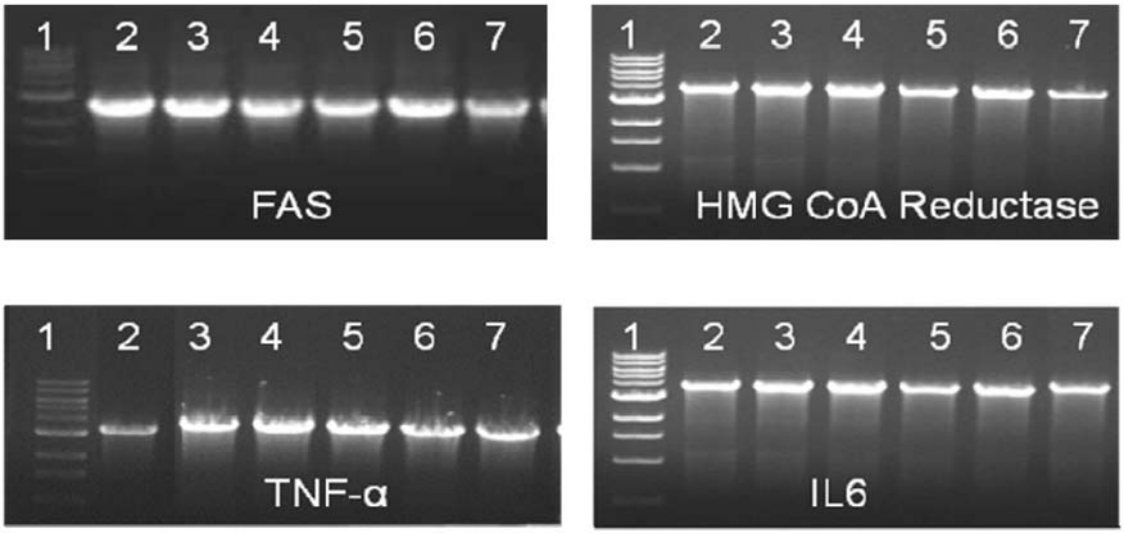

Figure 7. Effect of phytochemicals and oral hypoglycemic drugs on the expression of fatty acid synthase (FAS), HMG-CoA reductase, TNF-α and IL-6 transcripts in 3T3-L1 adipocytes shown by reverse transcription polymerase chain reaction. Lane 1 = 100 bp marker, lane 2 = control (no treatment), lane 3 = 2,4-thiazolidinedione, lane 4 = metformin, lane 5 = berberine, lane 6 = arecoline, lane 7 = vanillic acid.

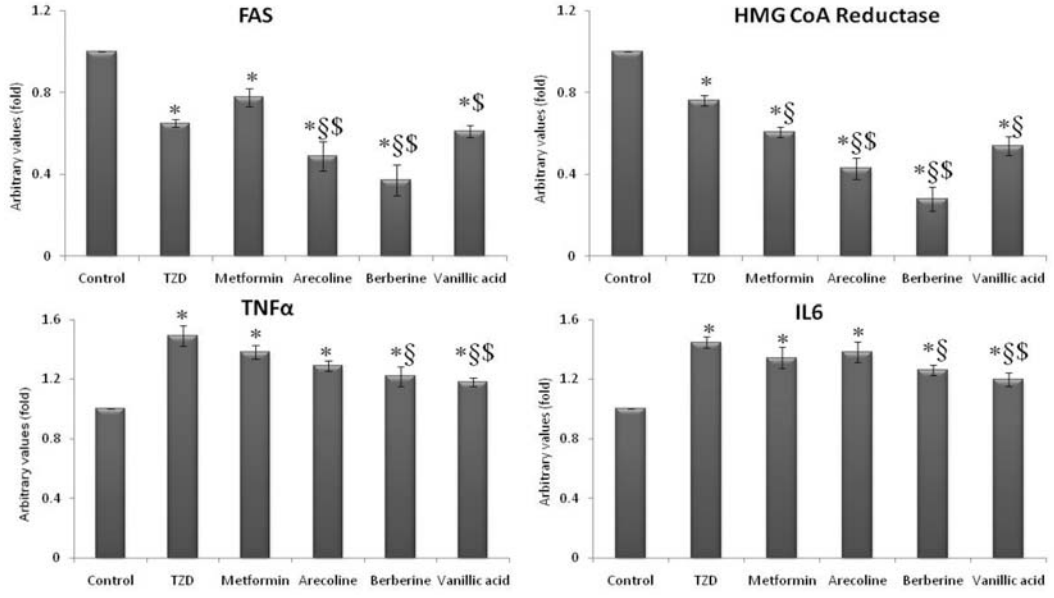

Figure 8. Densitometric scanning of fatty acid synthase (FAS), HMG-CoA reductase, TNF-α and IL-6 transcripts in the presence of commercial drug and natural products. Bars represent mean ± SD of three independent experiments. *$p < 0.05$ when compared with control; §$p < 0.05$ when compared with 2,4-thiazolidinedione; \$$p < 0.05$ when compared with metformin. Are, arecoline; Ber, berberine; Met, metformin; TZD, 2,4-thiazolidinedione; Van, vanillic acid.

Table 2. Absorption, distribution, metabolism and excretion and drug-likeness properties of the compounds calculated using QikProp® software.

	QPlogBB	QPlogS	Percent human oral absorption	Rule of five violations	Rule of three violations
TZD	−0.491	−0.634	66.044	0	0
Metformin	−1.184	−0.595	63.021	0	0
Berberine	0.401	−3.701	100	0	0
Arecoline	−0.736	−1.308	70.022	0	0
Vanillic acid	−0.889	−1.405	66.816	0	0

TZD, 2,4-thiazolidinedione.

respectively) when compared with the control. The reductions in the expression of both the genes by these compounds were statistically significant at 0.05% level.

Figure 7 shows the effects of these phytochemicals on the expression of the genes for two proinflammatory cytokines: TNF-α and IL−6. All the phytochemicals significantly ($p < 0.05$) increased the expression of TNF−α and IL−6 when compared with control (Figure 8).

Absorption, distribution, metabolism and excretion and drug-likeness properties of the phytochemicals

The drug-likeness and ADME properties of these compounds calculated theoretically are listed in Table 2. There were no violations of Lipinski's rule of five or Jorgensen's rule of three by any of the compounds. The percent human oral absorption for all the compounds, except berberine, was in the range 63–70% (for berberine ~100%), indicating that their bioavailability is in the recommended range. High human oral absorption values indicate that the compounds will permeate the cells through passive diffusion. Similarly, the compounds with fewer (and preferably no) violations of the rule of three or the rule of five are more likely to be orally available [Duchowicz *et al.* 2007]. The observed ranges for the QPlogBB and QPlogS were −1.184 to 0.401 and −3.701 to −1.405, respectively, which lie well within the recommended ranges. On the basis of these five descriptors it can be concluded that all the phytochemicals have reasonably good ADME properties and they also possess drug-like properties.

Discussion and conclusion

Insulin resistance and obesity are the main hallmarks of type 2 diabetes. Adipose tissues are one of the most important targets for the action of insulin and they play a major role in maintaining whole-body energy homeostasis. The 3T3-L1 cell line is routinely used in signaling studies and is considered to demonstrate all the features of adipocytes. It is a well-established model system in which to study the regulation of glucose transport, since muscle is the site that utilizes and disposes of glucose [Patel *et al.* 2008].

The main problem in the case of diabetes is insufficient utilization of blood glucose, which may be caused by either impaired glucose transport or reduced GLUT4 translocation. PPARγ, PI3K and AMPK play an important role in glucose transportation inside the cells. PI3K is the key molecule in the insulin signaling pathway, and its complete inhibition abolishes glucose uptake. Glucose uptake in cultured cells is routinely determined by using nonmetabolizable radioactive hexoses, including 3-methlyglucose or 2DG labeled with tritium [Sasson *et al.* 1993]. Assaying the uptake of the latter is more convenient than assaying with the former because 2DG is converted into a stable and impermeable derivative, 2DG6P, through phosphorylation by hexokinase or glucokinase [Sokoloff *et al.* 1977].

The increased triglyceride levels seen in diabetes are an important cause of secondary complications such as cardiovascular diseases and stroke. Both the OHDs and the phytochemicals significantly reduced the triglyceride concentration in 3T3-L1 adipocytes. Hence, it could be assumed that they can also decrease the secondary complications arising from high lipid levels.

All three phytochemicals increased 2DG uptake in a dose- and time-dependent manner, shown by both the methods we used. Berberine and arecoline showed synergy with TZD and metformin and boosted the effects of these two commercial

drugs on the uptake of 2DG. It was observed that the use of one of these phytochemicals might allow the reduction of the dose of the OHDs required to achieve the required glucose uptake. A reduction in the dose of OHDs could also lead to a reduction in side effects and toxicity. Conversely, vanillic acid exhibited antagonistic behavior in combination with metformin and additive or indifferent behavior with TZD.

The expression of *GLUT4*, *PPARγ* and *AMPK* increased when the commercial drugs were used. The activation of *PPARγ* by its agonist or an increase in the expression of *PI3K* and *AMPK* increased glucose uptake by 3T3-L1 adipocytes. Berberine and vanillic acid probably increase glucose uptake via the *AMPK* pathway and arecoline increases glucose via the *PPARγ* pathway.

All the compounds significantly reduced the expressions of fatty acid synthase, a regulatory hormone of fatty acid synthesis, and HMG-CoA reductase, a rate-limiting enzyme of cholesterol synthesis. These lipids have an important role in insulin resistance and the secondary complications of diabetes. Reductions in fatty acid and cholesterol synthesis might reduce insulin resistance and the chances of developing type 2 diabetes.

These three phytochemicals seemed to possess (based on theoretical calculations) good ADME and drug-likeness properties and hence could be used as drugs to partly replace commercial hypoglycemic agents. All three phytochemicals considered in the current study appeared to have good theoretical oral bioavailability. Based on the MTT assay it could be assumed that the concentrations of the natural products and commercial drugs chosen here were well below their cytotoxic level for the cells.

The results of the present study indicate that combination of arecoline or berberine with TZD or metformin act in synergy to increase 2DG uptake by 3T3-L1 adipocytes. They provide an opportunity to reduce the dose of both OHDs, which could help in minimizing their adverse effects and achieving enhanced therapeutic effects. Vanillic acid exhibited additive or antagonistic effects with both the OHDs. The phytochemicals might also reduce secondary complications. Proper precautions and care should be taken to avoid the severe hypoglycemia that might occur with use of combinations of these phytochemicals and OHDs. Although these phytochemicals are very safe, research on their long-term usage should be conducted.

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Conflict of interest statement

The authors declare no conflicts of interest in preparing this article.

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