JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Interaction of Cinnamic Acid Derivatives with Commercial Hypoglycemic Drugs on 2-Deoxyglucose Uptake in 3T3-L1 Adipocytes

Pranav Kumar Prabhakar and Mukesh Doble*

Department of Biotechnology, Indian Institute of Technology, Madras, Chennai 600 036, India

Supporting Information

ABSTRACT: Hydroxycinnamic acid derivatives are naturally occurring substances found in fruits, vegetables, and flowers and are consumed as dietary phenolic compounds. The effect of cinnamic acid, ferulic acid, *p*-coumaric acid, eugenol, chlorogenic acid, and caffeic acid, alone and in combination with two commercial oral hypoglycemic drugs (OHD), namely, thiazolidinedione (THZ) and metformin, on the uptake of 2-deoxyglucose (2DG) by 3T3-L1 adipocytes is studied. All of the phytochemicals other than cinnamic acid show synergistic interaction in 2DG uptake with both of the OHDs. THZ (20 μM) in combination with ferulic acid (25 μM) or *p*-coumaric acid (25 μM) increases 2DG uptake by 7- or 6.34-fold, respectively, with respect to control, whereas metformin (20 μM), along with ferulic acid (25 μM) or cinnamic acid (25 μM), increases 2DG uptake by 6.45- or 5.87-fold, respectively, when compared to control. Chlorogenic and cinnamic acids increased the expression of PPARγ, whereas other hydroxycinnamic acids enhanced the expression of PI3K, indicating different mechanisms of action between these compounds. These phytochemicals were able to reduce the expressions of the fatty acid synthase and HMG CoA reductase genes, indicating that they may be able to reduce the secondary complications caused by the accumulation of lipids. These studies suggest that hydroxycinnamic acid derivatives may be beneficial for the treatment of diabetes mellitus. They may act as a supplement with commercial drugs and may reduce the secondary complications caused by OHDs.

KEYWORDS: synergy, combination index, isobologram, ADME, cinnamic acid, 2-deoxyglucose

INTRODUCTION

Diabetes is recognized as a group of heterogeneous disorders with the common elements of hyperglycemia and glucose intolerance, due to insulin deficiency, impaired effectiveness of insulin action, or both.[1] According to the World Health Organization (WHO), diabetes mellitus (DM), including all complications, ranks as the third leading cause of death. The diabetic population in the world was estimated to have doubled from 110 million in 1994 to 220 million in 2010.[2] There is a need for new hypoglycemic agents that will have therapeutic efficacy as well as fewer side effects. There are reports of managing diabetes with medicinal plants. Dietary intake from plant food and their ingredients could be a more effective strategy for the management of DM because of the likelihood of high compliance and because they are largely free from side effects, have better effectiveness, act on multiple target sites, and are of relatively low cost. Hence, new chemical compounds from natural products have been explored for possibly safer antidiabetic agents[3,4] that will have fewer side effects. Also, if the therapeutic concentration of the oral hypoglycemic drug (OHD) could be partly reduced by replacing it with a phytochemical, the side effects caused by the former could be decreased to a large extent.[5] For such a therapeutic strategy one has to consider the interaction between the OHD and the phytochemical. In addition, one has to understand the metabolism and bioavailability of the phytochemicals.

Phenolic compounds have been of considerable interest to consumers and food manufacturers for several reasons.[6,7] These compounds are widely distributed in the plant kingdom and are present in considerable amounts in fruits, vegetables, and beverages in the human diet.[8] Recent papers have documented that the consumption of phenolic compounds is associated with the prevention and reduced risk of several degenerative diseases including atherosclerosis, cardiovascular complications, and cancer.[9] Hydroxycinnamic acids (Figure 1; Table 1) are phenolic compounds and form one of the largest and most ubiquitous groups of plant metabolites, with many structures already identified. They possess a variety of pharmacological properties including hepatoprotective,[10] antimalarial,[11] antioxidant,[12] and antityrosinase activities.[13] It is observed that administration of ferulic acid helps to enhance the antioxidant capacity of diabetic mice by reducing the formation of free radicals and increasing the activities of antioxidative enzymes including superoxide dismutase, catalase, and glutathione peroxidase.[14] *p*-Hydroxycinnamic acid markedly reduces plasma cholesterol and hepatic lipids in high cholesterol fed rats.[15] This action may benefit those diabetic patients who are at the risk of developing chronic complications related to cerebrovascular, cardiovascular, and peripheral vascular diseases.

Earlier investigation revealed that ferulic acid, chlorogenic acid, and eugenol synergistically interact with OHDs in the uptake of 2-deoxyglucose (2DG) in L6 myotubes.[5] Eugenol interacts synergistically with hydrophilic antibiotics such as ampicillin, chloramphenicol, vancomycin, and polymixin and exhibits enhanced antibacterial activities.[16] An experiment performed with human blood has shown that the concentration of eugenol used

Received: April 19, 2011
Revised: August 23, 2011
Accepted: August 26, 2011
Published: August 26, 2011

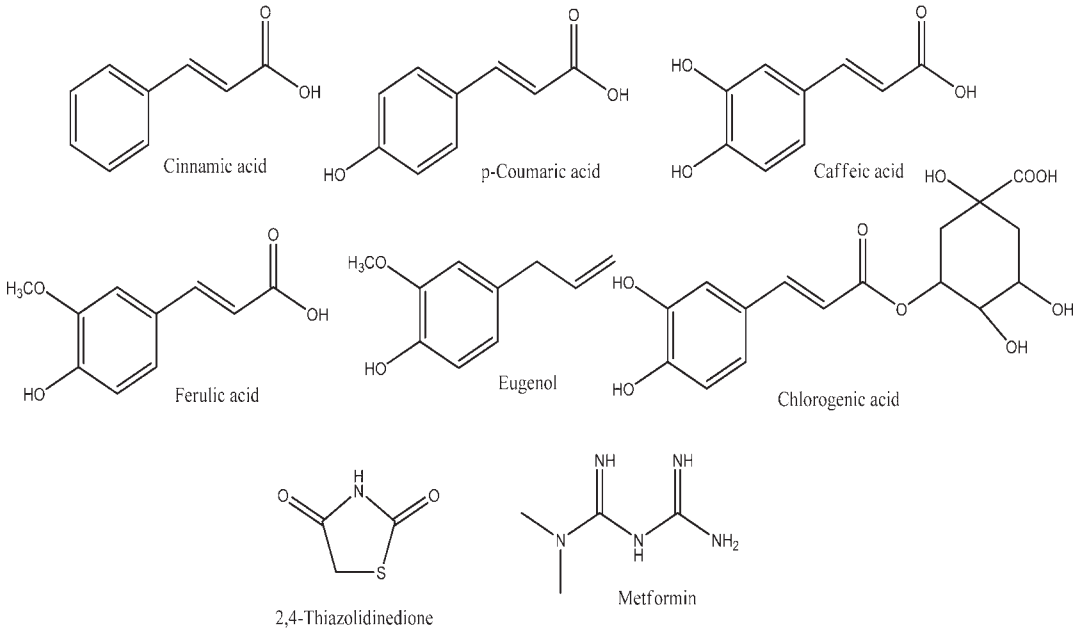

Figure 1. Structures of hydroxycinnamic acid derivatives and commercial oral antidiabetic drugs.

Table 1. Phytochemicals, Their Source Plants, and Activities

sample	phytochemical	plant sources	other activities	source and content (per serving)
1	caffeic acid	*Adonis vernalis* L.	analgesic, antioxidants	100 g of kiwi contains 60−100 mg
2	chlorogenic acid	coffee bean, *Flos lonicerae*	antitumor, antihistaminic	200 g of cherry contains 36−230 mg; 200 mg of coffee contains 96 mg
3	cinnamic acid	*Cinnamomum verum*	aldose reductase inhibitor	100 g of blueberry contains 200−220 mg
4	*p*-coumaric acid	*Arachis hypogaea, Allium sativum*	antioxidant, anticancer	200 g of plum contains 28−230 mg
5	eugenol	*Eugenia aromatica*	antibacterial, antiinflammatory	100 g of clove oil contains 0.45−0.87 g
6	ferulic acid	*Ferula communis*	antioxidant, anticancer	200 g of tomato contains 220−336 mg

in combination studies is well below its toxic level.[16] Plants containing these phytochemicals have been used in traditional Chinese and Asian medicine for treatment of cancer and hence may be free from toxicity and long-term side effects.[17]

Several studies have demonstrated the antidiabetic activity of cinnamic acid derivatives; however, the effect of combination of these cinnamic acid derivatives and OHDs is yet to be fully understood. The present study is aimed at addressing this lacuna. The effect of these phytochemicals on 2DG uptake alone and in combination with two OHDs by 3T3-L1 adipocytes is examined. The expressions of genes involved in the insulin cascade and in the secondary complications were also monitored. A nonradioisotopic enzymatic assay is used to estimate the uptake of 2DG by the cells.[18]

MATERIALS AND METHODS

Materials. 3T3-L1 adipocytes, derived from mouse embryonic fibroblast, were purchased from NCCS (Pune, India). Dulbecco 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, rezazurine, ATP, $NADP^+$, and all of the primers were obtained from Sigma-Aldrich (St. Louis, MO). THZ and BSA were purchased from Himedia Laboratories Pvt. Ltd. (Mumbai, India), and metformin and DMSO were purchased from Merck (Darmstadt, Germany). All other chemicals were proccurred from SRL Pvt. Ltd. (Mumbai, India). Medox easy spin column total RNA Miniprep super kit was purchased from Medox Biotech India Pvt. Ltd. (Chennai, India) and used for the total RNA extraction. RobusT I RT-PCR kit was purchased from Finzymes (Espoo, Finland). All of the primers were purchased from Bioserve (Hyderabad, India) and plasticwares from Tarson Products Pvt. Ltd. (Kolkata, India).

Determination of 2DG. 2DG is more convenient to use than glucose itself because it is phosphorylated to a stable and impermeable derivative, 2-DG-6-phosphate, by hexokinase or glucokinase. 2DG is estimated by an enzymatic diaphorase−NADPH-amplifying system assay as previously reported.[19] One hundred microliters of 2DG solution of different concentrations (0.25, 0.5, 1, 2, 5, and 10 μM) was dispensed into each well of a 24-well plate. After the addition of 300 μL of a reaction cocktail, consisting of 50 mM TEA (pH 8.1), 0.02% of BSA, 50 mM KCl, 0.5 mM $MgCl_2$, 670 μM ATP, 0.12 μM $NADP^+$, 25 μM resazurin sodium salt, 5.5 units/mL hexokinase, 16 units/mL G6PDH, and 1 unit/mL diaphorase, the mixture was incubated for 90 min. This cocktail was prepared just before the assay from the refrigerated stock solutions. After incubation, the fluorescence at 590 nm with excitation at 530 nm was measured with an FP-6500 research grade fluorescence spectrometer (M/s. Jasco International Co. Ltd., Japan) to detect the conversion of resazurin to resorufin. The amount of resorufin formed, if the reaction goes to completion, should be stoichiometrically equivalent to the amount of 2DG uptake. A standard curve was initially prepared between

Table 2. Primers Used for RT-PCR

gene	sequence	orientation
GLUT-4	5′-CCAGCCTACGCCACCATAG-3′	forward
	5′-TTCCAGCAGCAGCAGAGC-3′	reverse
PPAR-γ	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
HMCoA 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

fluorescence readings with different concentrations of 2DG in the well of the culture plate without the cells.

Cell Culture of 3T3-L1 Adipocytes. 3T3-L1 adipocytes (3.5×10^5) were grown in each well of a 24-well plate in DMEM supplemented with 10% of fetal bovine serum and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin) at 37 °C in a humidified atmosphere composed of 5% CO_2 in air. The medium was changed every third day. For the cell differentiation, the 3T3-L1 adipocytes were transferred to DMEM having 2% of FBS for 4−6 days postconfluency. The extent of differentiation was established by observing the formation of elongated and multinucleated 3T3-L1 adipocytes. These differentiated cells were used for further studies.

MTT Assay. The viability of the cells was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as described in the literature.[19] This assay is based on the reduction of MTT into purple formazan pigment by the mitochondrial succinate−tetrazolium reductase system.[20] The cells were seeded to 5×10^4 cells/mL density and incubated with both the phytochemicals and OHDs individually at various concentrations (0, 10, 20, 50, and 100 μM) for 24 and 48 h. After the specified time, the medium was replaced with MTT solution (0.5 mg/mL in PBS) and incubated for 4 h at 37 °C. The formazan formed inside the cells was dissolved in 0.04 N HCl taken in isopropanol, and the absorbance was measured spectrophotometrically at 595 nm with a Spectramax Plus384 spectrophotometer (Molecular Devices, Sunnyvale, CA). The number of viable cells is directly proportional to the production of formazan.

Oil Red O Method (Differentiation Assay). Accumulation of triglyceride inside the cells is determined by the staining of neutral lipids with Oil Red O (ORO) dye.[21] After the appropriate incubation with the test compounds, 3T3-L1 adipocytes were fixed with perchloric acid. Later the cells were washed with distilled water and then were submerged in wells containing ORO dissolved in propylene glycol (2 mg/mL). The wells were then washed with PBS three times after overnight incubation at room temperature. ORO was extracted using isopropanol for 10 min at room temperature. The absorbance was measured at 490 nm with a V-670 research grade UV−vis spectrometer (M/s. Jasco International Co. Ltd.) and blanked to cell-free well.

2-Deoxyglucose Uptake Assay. The differentiated 3T3-L1 adipocytes were starved of serum for 4 h in DMEM and then were rinsed twice in (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). They were incubated with 350 μL/well of DMEM and 2% FBS in the presence of the test compounds (both phytochemicals and OHDs) for specified times. Cells were washed twice with KRPH buffer containing 0.1% BSA and were incubated with the same buffer containing 1.5 mM 2DG and 0.1% BSA for 30 min at 37 °C in a 5% CO_2 atmosphere. After incubation, they were rinsed with the same buffer containing 0.1% BSA and 50 μL of NaOH (0.1 N). Fifty microliters of HCl (0.1 N) was added to neutralize the alkalinity in the wells followed by the addition of 50 μL of 150 mM TEA buffer (pH 8.1). The fluorescence is measured by the enzymatic method as described above, and the reading corresponds to the concentration of 2DG inside the cell.

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

Synergistic interaction of a phytochemical with a commercial OHD is ascertained on the basis of a calculated parameter termed the combination index (CI).[22] It is estimated as

$$\text{combination index (CI)} = \left[\frac{C_A}{IC_A}\right] + \left[\frac{C_B}{IC_B}\right]$$

where C_A and C_B are the concentrations of compounds A and 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 compound required individually to achieve the same effect. A CI value of less than, equal to, and more than 1, indicates synergy, additivity, and antagonism, respectively, between these two compounds.

The presence of synergy between two compounds is also visually represented in the form of an isobologram. The *X*- and *Y*-axes of the isobologram are the concentrations of 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 required to achieve the same level of 2DG uptake is termed the "line of additive". If the combination curve is well below this straight line (based on 95% confidence band), then the curve is termed the "synergistic concentration".[23]

Reverse Transcriptase-Polymerase Chain reaction (RTPCR). RTPCR of the genes involved in the insulin cascade (namely, GLUT4, PPARγ, and PI3K), adipogenic enzymes (fatty acid synthase and HMG CoA reductase), and pro-inflammatory cytokines (tumor necrosis factor α and IL-6) was performed as described in the literature.[24] The total RNA from 3T3-L1 adipocytes was isolated after incubation with the phytochemical or the commercial OHD for a specified time with the help of a total RNA Miniprep super kit as per the manufacturer's instructions. RTPCR was carried out to obtain the cDNA with 5 U/μL AMV reverse transcriptase along with 20 pg of template RNA. The primers used are listed in Table 2. PCR reaction mix consists of 10× PCR buffer, 10 mM of each of the dNTP, 10 pmol of paired primers, 2 units of DNA polymerase, and distilled water, to make up to a total volume of 50 mL. This mixture was overlaid with mineral oil and placed in a PCR thermal cycler for 30 cyclic reactions in PTC-200 DNA Engine thermal cycler (MJ Research, South San Francisco, CA). The products were run on 1.5% agarose gel, stained with ethidium bromide, and photographed. The densities of the bands were obtained by scanning the gels with GelDock (Bio-Rad, Hercules, CA). The density of the control sample was considered as 1, and those of the others were expressed in multiples of this.

Absorption, Distribution, Metabolism, and Excretion (ADME) Property Calculation. The physiological process involved in the ADME of a compound is an important determinant of its therapeutic efficacy. There are many commercial softwares that can theoretically estimate these parameters for a given compound. The structures of all the phytochemicals and the two OHDs were drawn using Hyperchem software (HyperCube Inc., Gainesville, FL), and their minimum energy conformations were obtained using MM+ force field. QikProp (Schrödinger Inc., Portland, OR) was used for calculating the ADME and drug-like properties of these compounds. These descriptors are QPlogBB (predicted brain/blood partition coefficient), percent human-oral absorption (predicted human oral absorption on 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, QPlogPo/w (predicted octanol/water partition coefficient) < 5, hydrogen bond donor ≤ 5, hydrogen bond acceptor ≤ 10"),[25] and number of violations of Jorgensen's rule of three (the rule is "QPlogS > −5.7, QPPCaco (predicted apparent Caco-2 cell permeability) > 22 nm/s, number of primary metabolites < 7").[26,27] The recommended range for QPlogBB is from −3.0 to 1.2, that for percent human-oral absorption is 25−80%, that for QPlogS is from −6.5 to 0.5, and permitted violation of the rules of five and three are 1 and 0, respectively. These descriptors described the ADME, drug-like properties, and oral bioavailability of the compounds.

Statistical Analysis. All of the experiments were performed in triplicate, and the results were expressed as the average ± standard deviation. Differences among treatments were determined using ANOVA and Tukey's posthoc multiple-comparison test considering $p < 0.05$ to be significant with SPSS software (SPSS South Asia Pvt. Ltd., Bangalore, India).

RESULTS

MTT Assay. 2DG is a glucose molecule that has the 2-hydroxyl group replaced by hydrogen, so it cannot undergo further glycolysis. Because it hampers cell growth, its use as a therapeutic for tumors has been suggested. The toxicity of 2DG at various concentrations was studied (results not shown), and it was found to be toxic to 3T3-L1 adipocytes above a concentration of 1.5 μM; hence, further experiments were performed at this value.

Table 3. Effects of Phytochemicals and OHDs on Cell Viability in 24 h Treatment

	1 μM	5 μM	10 μM	20 μM	50 μM	100 μM
THZ	97.13	92.19	89.11	85.59	79.66	75.92
metformin	94.75	90.25	87.69	82.29	76.04	70.41
caffeic acid	99.03	93.76	90.15	89.65	85.39	79.46
chlorogenic acid	98.31	92.13	88.65	84.07	80.65	72.34
cinnamic acid	96.07	90.04	90.59	86.25	82.92	75.12
coumaric acid	97.10	93.62	88.58	82.55	76.43	72.75
eugenol	98.81	93.68	90.91	85.24	79.48	71.12
ferulic acid	97.05	91.72	86.93	82.43	79.10	73.10

The MTT assay, which measures the mitochondrial reductase enzyme activity, is a measure of viable cells. At a concentration of 50 μM of the phytochemicals, 75−85% of the cells were viable when compared to control after 24 h of incubation (Table 3). Therefore, all experiments were performed below this concentration.

Differentiation Assay (TG Content). The phytochemicals (at 25 μM) induce the differentiation of 3T3-L1 adipocytes but less than the control and THZ (Figure 2). Quercetin at 100 mM suppressed the accumulation of TG in mature 3T3-L1 cells. The TG value was 40% when compared to untreated cells ($p < 0.01$). The TG contents when treated with THZ and metformin are 84 and 67% ($p < 0.01$), respectively. The difference in TC contents after treatment with metformin, caffeic acid, chlorogenic acid, eugenol, and ferulic acid were not significantly different from one another (at $p < 0.05$). Accumulation of TG in cells treated with phytochemicals is less when compared to the cells treated with THZ. The accumulation of TG in cells treated with ferulic acid and *p*-coumaric acid are 72 and 53%, respectively.

Dose- and Time-Dependent 2DG Uptake in 3T3-L1 Adipocytes. All of the compounds increase the uptake of 2DG by 3.5×10^5 differentiated 3T3-L1 adipocytes in dose- and time-dependent manners. Metformin increased 2DG uptake by 2.98-fold (when compared to control) at a concentration of 15 μM after 4 h of incubation, whereas THZ increased it by 3.26-fold at 10 μM concentration after 3 h of incubation (Figure S1A of the Supporting Information). In the case of THZ and phytochemicals the maximum 2DG uptake reached a maximum at the fourth hour (Figure S1B of the Supporting Information), whereas in the case of metformin the 2DG uptake reached maximum at the third hour.

Interaction of Cinnamic Acid Derivatives with OHD. Different concentrations of the natural products in combination with various concentrations of OHD (2.5, 5, 10, 20, and 25 μM) were tested to determine the effect of combination on the uptake of 2DG.

All of the phytochemicals, except cinnamic acid, showed synergistic behavior with both the OHDs in the uptake of 2DG, whereas cinnamic acid exhibited an additive effect. THZ (20 μM) in combination with ferulic acid (25 μM), eugenol (50 μM), chlorogenic acid (25 μM), caffeic acid (25 μM), and *p*-coumaric acid (25 μM) increased 2DG uptake by 7-, 6.8-, 6.7-, 6.5-, and 6.34-fold, respectively, with respect to control. Metformin (20 μM) with ferulic acid (25 μM), eugenol (25 μM), chlorogenic acid (25 μM), *p*-coumaric acid (25 μM), caffeic acid (25 μM), and

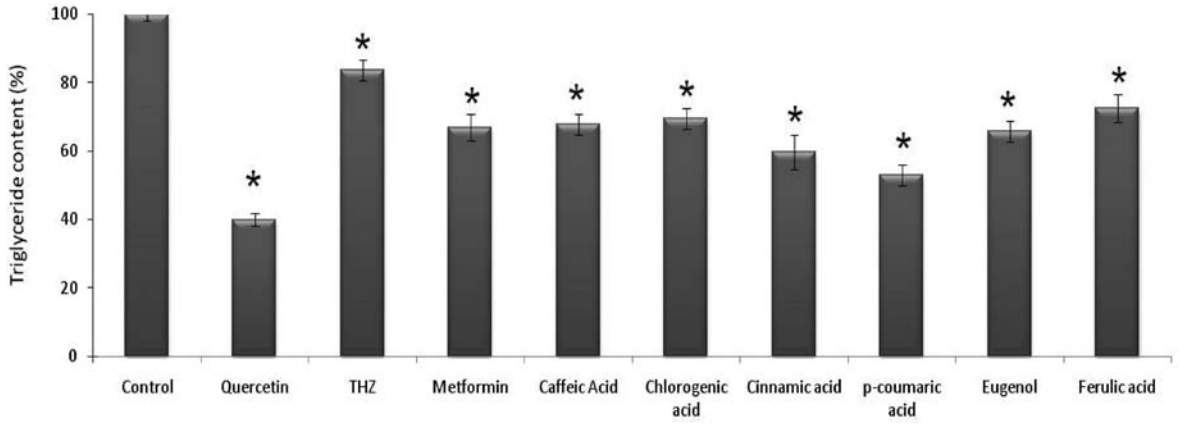

Figure 2. Effect of various treatments on the triglyceride levels in 3T3-L1 adipocytes (50 μM concentrations and 4 h of incubation).

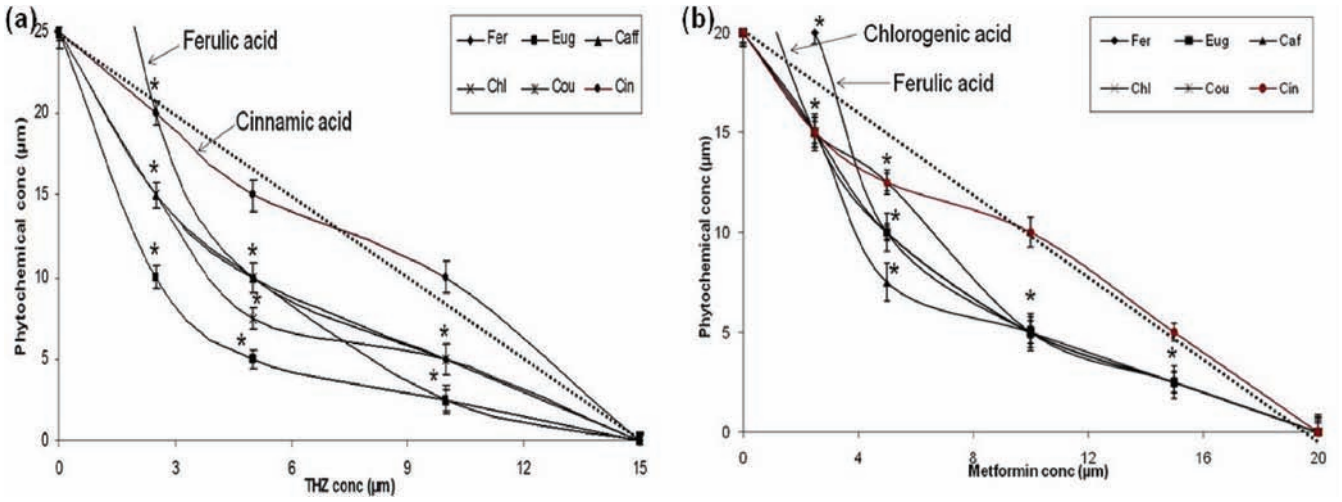

Figure 3. Isobologram representing the interaction of phytochemicals with (a) THZ for a 2DG uptake of 263 ng/3.5 $\times$ 10^5 3T3-L1 adipocytes and (b) metformin for a 2DG uptake of 237 ng by 3.5 $\times$ 10^5 3T3-L1 adipocytes ((*) $p < 0.05$ when compared to control).

cinnamic acid (25 μM) increased 2DG uptake by 6.5-, 6.4-, 6.2-, 6.1-, 6.3-, and 5.9-fold, respectively, when compared to control.

These interactions are represented as isobolograms as well as combination index. The isobolograms relating the concentrations of various phytochemicals with THZ to achieve a 2DG uptake of 263 ng/3.5 $\times$ 10^5 3T3-L1 adipocytes (i.e., 3.29-fold increase in 2DG uptake, which is the maximum uptake with THZ alone) are shown in Figure 3a. Similarly, the isobolograms relating the concentrations of various phytochemicals with metformin to achieve a 2DG uptake of 236 ng/3.5 $\times$ 10^5 3T3-L1 adipocytes (i.e., 2.95-fold increase in 2DG uptake, which is the maximum uptake with metformin alone) are shown in Figure 3b. Cinnamic acid shows additive behavior with both the OHDs (the curve is close to the line of additive), whereas the combination curves for other phytochemicals and OHDs are well below the line of additive, indicating synergy. The combination indices for cinnamic acid and THZ at various ratios are almost equal to 1, indicating additive behavior, whereas the CI for the other five phytochemicals and THZ at different ratios of these two are <1, indicating synergy (Tables S1−S6 of the Supporting Information). Similar observations are made between metformin and the phytochemicals (Tables S7−12 of the Supporting Information). In the case of all the synergistic combinations, phytochemicals reduce the dose of THZ to obtain a 2DG uptake of 263 ng/3.5 $\times$ 10^5 3T3-L1 adipocytes (i.e., 3.29-fold increase in 2DG uptake). For example, 10 μM ferulic acid reduces the dose of THZ by one-fourth to achieve the 3.29-fold increase in 2DG uptake, and 15 μM chlorogenic acid reduces the dose of THZ by one-sixth for the same 2DG uptake.

Also, 10 μM eugenol or 10 μM ferulic acid reduces the dose of metformin by one-fourth to achieve a 2.95-fold increase in 2DG uptake, and 5 μM chlorogenic acid reduces the dose of metformin by half for the same 2DG uptake.

Reverse Transcriptase Polymerase Chain Reaction. Adipocytes were treated with the test compounds alone, and the expressions of genes involved in the insulin cascade (GLUT4, PPARγ, and PI3K), adipogenic enzymes (fatty acid synthase, HMG CoA reductase), and pro-inflammatory cytokines (tumor necrosis factor α and IL-6) were investigated by RTPCR. All of the phytochemicals, except chlorogenic and cinnamic acid, significantly increased the expressions of GLUT4 and PI3K, whereas those two acids increased the expressions of GLUT4 and PPARγ, which indicates that the mechanism of action of these two and the rest of the derivatives are different. On this basis we can hypothesize that the mechanism of action of chlorogenic acid and cinnamic acid might be via PPARγ-dependent pathways and that

the other compounds achieve this via PI3K pathways (Figure 4). Densitometry scanning of the expressions of these genes indicate (Figure 5) that maximum increase in the expressions of GLUT4 and PI3K was achieved by ferulic acid (3.22- and 3.23-fold), whereas THZ increases them by 3.35- and 1.78-fold, respectively, when compared to control (Tukey's posthoc multiple-comparison test, $p < 0.01$). Eugenol, *p*-coumaric acid, caffeic acid, cinnamic acid, and chlorogenic acid increase PI3K expression by 3.11-, 2.91-, 2.69-, 1.59-, and 1.53-fold, respectively. Maximum increase in PPAR gene is achieved with chlorogenic acid (2.7-fold) followed by cinnamic acid (2.11-fold) when compared to control, whereas the OHDs, THZ and metformin, increase it by 2.73- and 1.52-fold, respectively. Ferulic acid, *p*-coumaric acid, caffeic acid, and eugenol increase it by 1.57-, 1.24-, 1.51-, and 1.36-fold, respectively. The differences within the groups, except a few such as the effect of THZ with ferulic acid, metformin with cinnamic acid, metformin with *p*-coumaric acid, caffeic acid with chlorogenic acid, caffeic acid with cinnamic acid, caffeic acid with *p*-coumaric acid, and caffeic acid with eugenol for GLUT4 expression, are not significant (Tukey's posthoc multiple-comparison test, $p < 0.05$) (Tables S13 and S14 of the Supporting Information).

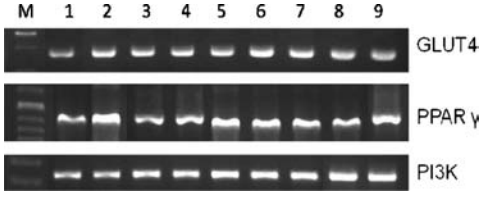

Figure 4. Effect of phytochemicals and OHDs on the expressions of GLUT4, PI3K, and PPARγ transcripts in 3T3-L1 adipocytes (as shown by RT-PCR). Lanes: M, marker; 1, control; 2, THZ; 3, metformin; 4, cinnamic acid; 5, *p*-coumaric acid; 6, caffeic acid; 7, eugenol; 8, chlorogenic acid; 9, ferulic acid.

Figure 6 shows the effects of these phytochemicals on the expressions of two pro-inflammatory cytokines, namely, TNF-α and IL-6 genes. They increase the expressions of both the genes ($p < 0.05$). These phytochemicals significantly decrease the expression of FAS as well as HMG CoA reductase, which indicates that they may reduce the secondary complications. Densitometry scanning shows that the reduction in the expressions of FAS and HMG CoA reductase is maximum with ferulic acid (73%) followed by eugenol (67%), chlorogenic acid (66%), *p*-coumaric acid (46%), cinnamic acid (46%), caffeic acid (43%), metformin (39%), and finally THZ (24%) when compared to the control. Cinnamic acid derivatives significantly ($p < 0.05$) increase the expressions of TNF-α and IL-6 when compared to control. The difference

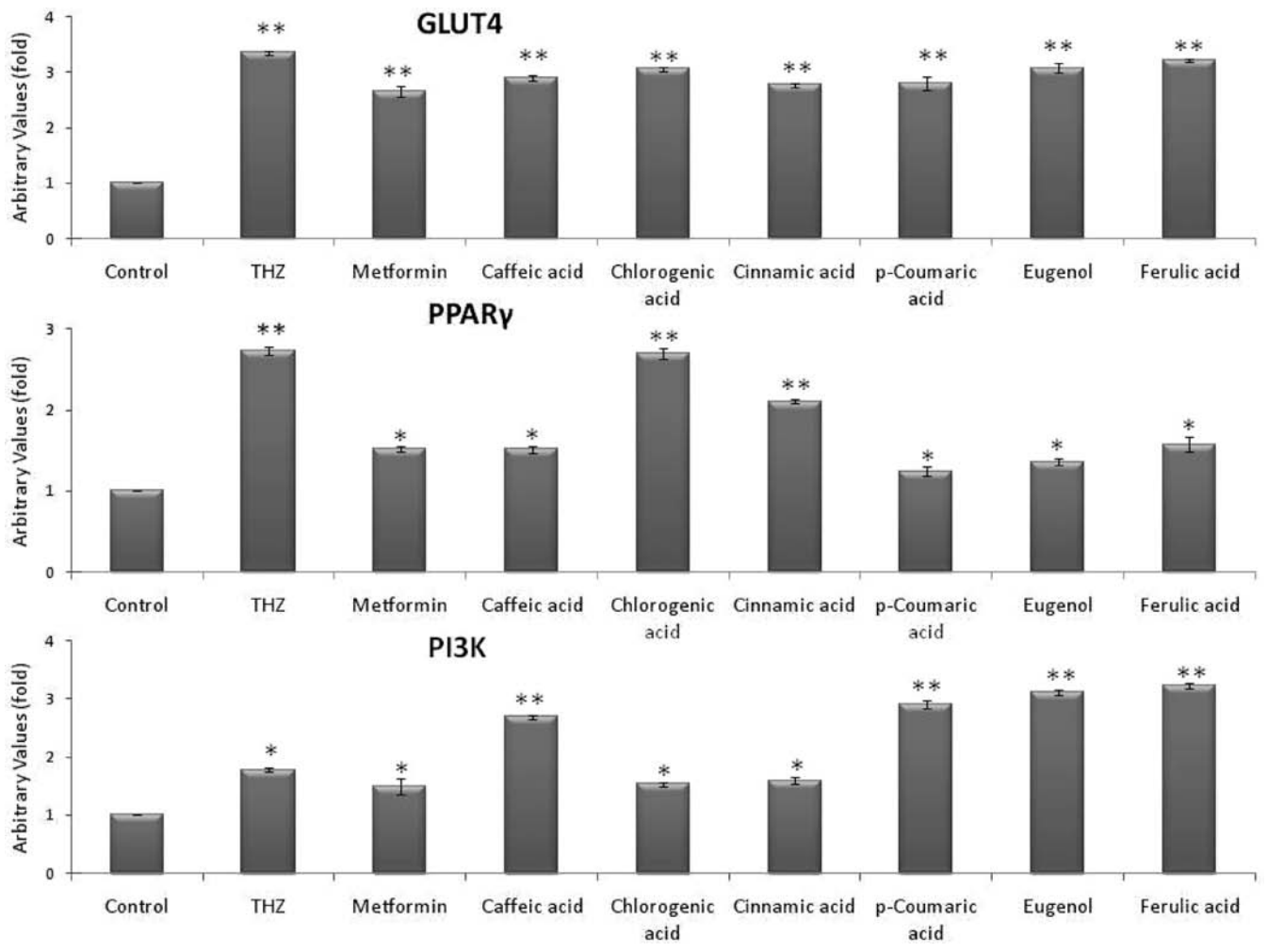

Figure 5. Densitometric scanning of GLUT4, PPARγ, and PI3K transcripts in the presence of commercial drug and natural products. Bars represent the mean ± SD of three independent experiments ((*) $p < 0.05$ and (**) $p < 0.01$ when compared to control).

between the groups was not statistically significant at the 95% level (Tukey's posthoc test) (Figure 7).

ADME and Drug-like Properties of the Hydroxycinnamic Acid Derivatives. The five descriptors that describe the drug-like and ADME properties of these compounds are listed in Table 4. The percent human oral absorption for all of the compounds, except chlorogenic acid, is in the range of 56−81% (for chlorogenic acid it is equal to 28%) and hence within the recommended range. If the percentage human oral absorption value is high, then passive diffusion will be predominant, particularly transcellular in its permeation. Similarly, the compounds with fewer (and preferably no) violations of the rules of three and five are more likely to be orally available.[27] None of the phytochemicals except chlorogenic acid and the two commercial drugs violate these two rules (chlorogenic acid violates one rule in both cases). The observed ranges for the QPlogBB and QPlogS are, respectively, from −0.889 to −0.058 and from 2.186 to −1.308. Therefore, on the basis of these five descriptors it can be concluded that all of the compounds have reasonably good ADME properties and possess drug-like properties.

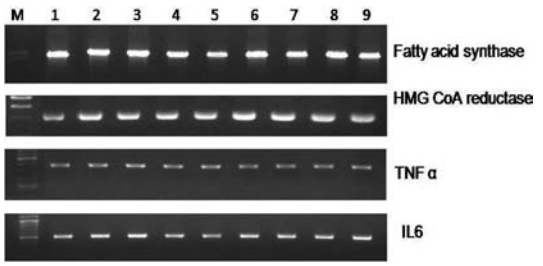

Figure 6. Effect of phytochemicals and OHDs on the expressions of fatty acid synthase, HMG CoA reductase, TNF-α, and IL-6 transcripts in 3T3-L1 adipocytes (as shown by RTPCR). Lanes: M, marker; 1, control; 2, THZ; 3, metformin; 4, cinnamic acid; 5, *p*-coumaric acid; 6, caffeic acid; 7, eugenol; 8, chlorogenic acid; 9, ferulic acid.

DISCUSSION

Insulin resistance and obesity are hallmarks of type 2 diabetes. Adipose tissues, as one of the targets for the action of insulin, play an important role in maintaining the whole body energy homeostasis. 3T3-L1 cells are routinely used in the signaling studies

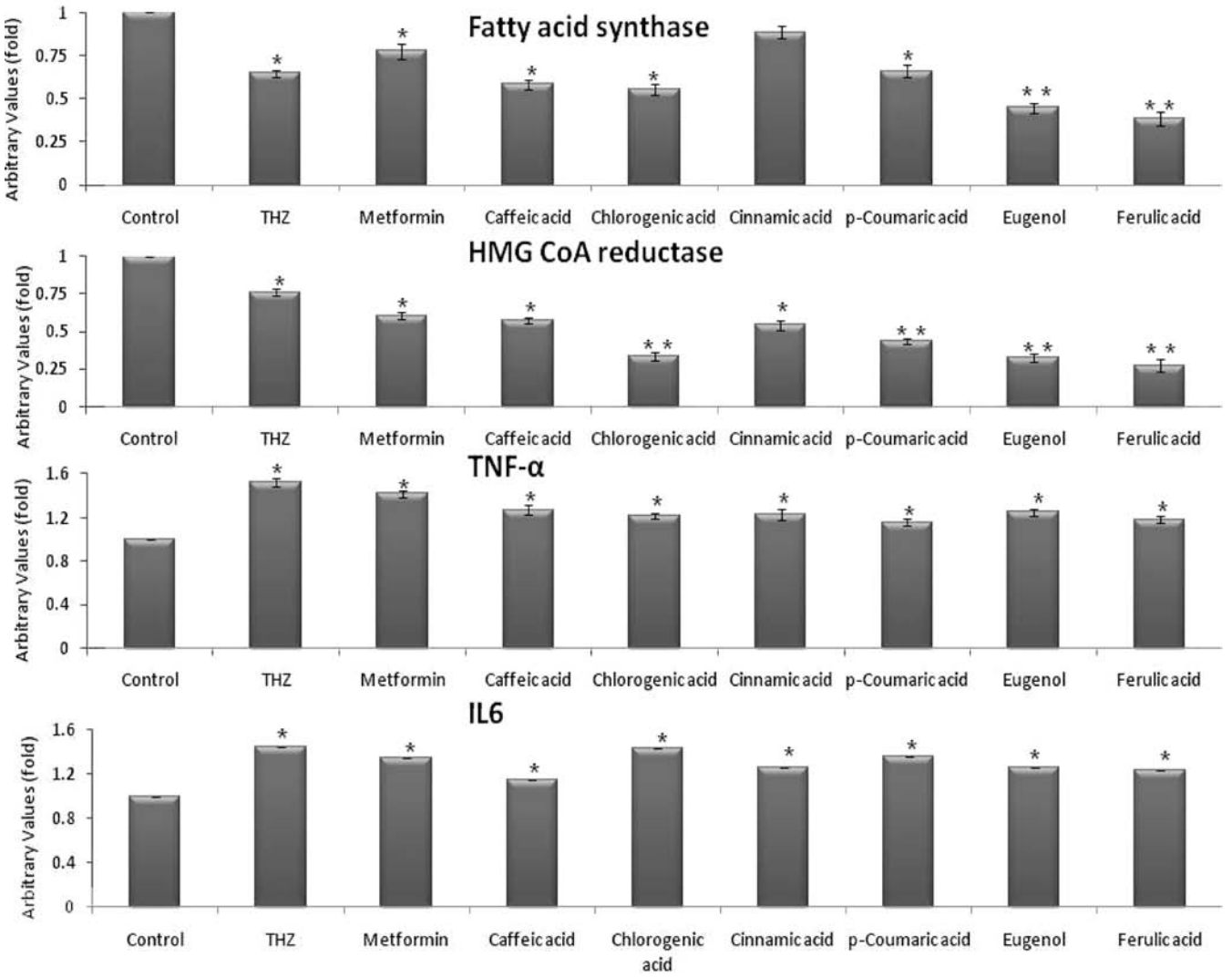

Figure 7. Densitometric scanning of fatty acid synthase, HMG CoA reductase, TNF-α, and IL-6 transcripts in the presence of commercial drug and natural products. Bars represent the mean ± SD of three independent experiments ((∗) $p < 0.05$ and (∗∗) $p < 0.01$ when compared to control).

dx.doi.org/10.1021/jf2015717 |*J. Agric. Food Chem.* 2011, 59, 9835–9844

and are regarded to demonstrate all of the features of adipocytes. 3T3-L1 adipocytes are a well-established model system to study the regulation of glucose transport and energy homeostasis.[28] The concentrations of the natural products and commercial drugs chosen here are well below the cytotoxic level of the cells.

The main problem in the case of diabetes is insufficient utilization of blood glucose, which may be caused by impaired glucose transport or reduced GLUT4 translocation. PPARγ and PI3K play very important roles in glucose transportation inside the cells. The latter 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 3MG or 2DG labeled with tritium.[29] Assaying the uptake of [3H]2DG is more convenient than assaying the uptake by other methods becausse it is converted to a stable and impermeable derivative, 2DG6P, through phosphorylation by hexokinase or glucokinase.[30]

The serum lipid has an important role in insulin resistance, and its increase is an important cause of secondary complications including cardiovascular diseases, stroke, etc., in diabetes. Both the OHDs and the phytochemicals significantly reduced the lipid concentration in the 3T3-L1 adipocytes. Hence, they can also decrease the secondary complications arising due to higher levels of lipids.

Table 4. ADME and Drug-like Properties Calculated by QikProp

	QPlogBB	QPlogS	% human oral absorption	rule of five violation	rule of three violation
THZ	−0.491	−0.634	66.044	0	0
metformin	−1.184	−0.595	63.021	0	0
chlorogenic acid	−3.365	−2.481	28.063	1	1
ferulic acid	−1.011	−1.751	70.59	0	0
coumaric acid	−0.251	2	67.595	0	0
cinnamic acid	0.026	1.073	80.981	0	0
caffeic acid	−0.484	2	55.729	0	0
eugenol	−0.058	−2.186	77.878	0	0

The current study indicates that eugenol, chlorogenic acid, ferulic acid, *p*-coumaric acid, cinnamic acid, and caffeic acid act in synergy with both of the OHDs (THZ and metformin), so these phytochemicals will boost the effects of these two commercial drugs. With the use of one of these phytochemicals, one would be able to reduce the dose of the OHD to achieve the same glucose uptake. Therefore, a part of the commercial drug could be replaced with the above-mentioned phytochemicals to get the same biochemical effect. A reduction in the quantity of the OHD could also lead to a reduction in the side effects and toxicity caused due to its excess usage.

The plot of the concentration of the phytochemicals and OHDs to achieve a certain amount of 2DG uptake is known as an isobologram. Isobolograms pictorially indicate the synergy and additive nature of the action of two compounds when they are used in combination. The combination index is another technique to elucidate the interaction between two compounds. Both of these methods indicate that, except for cinnamic acid, all five phytochemicals interact with THZ and metformin synergistically and enhance 2DG uptake.

There are two important pathways to activate the translocation of GLUT4 vesicles from cytoplasm to plasma membrane, that is, via PI3K and via PPARγ. The PPARγ pathway includes Grb, SOS, Ras, Raf, and MAP kinase, whereas the PI3K pathway includes PDK, Akb, protein kinase B, etc. (Figure 8). The expressions of PPARγ and GLUT4 increase when the commercial drugs are used. Activation of PPARγ by its agonist or PI3K increases the glucose uptake in 3T3-L1 adipocytes. PI3K is a downstream signaling molecule in the insulin cascade that enhances glucose uptake by the cell via the activation of GLUT4 translocation.[4] Four cinnamic acid derivatives, namely, ferulic acid, eugenol, *p*-coumaric acid, and caffeic acid, have significantly increased expressions of GLUT 4 and PI3K, whereas chlorogenic acid and cinnamic acid have significant effects on the expression of the PPARγ gene. These hydroxycinnamic acid derivatives act

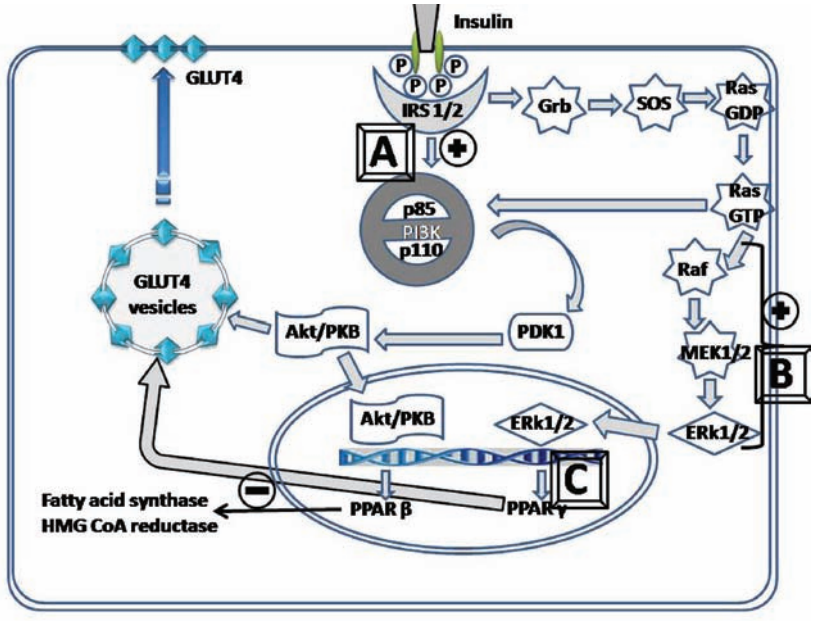

Figure 8. Mechanism of action of hydroxycinnamic acid derivatives and commercial drugs (A, ferulic acid, eugenol, *p*-coumaric acid, caffeic caid; B, chlorogenic acid, cinnamic acid; C, THZ; + indicates increase and − indicates decrease).

differently on the expressions of the down-regulators of the insulin cascade, so it could be hypothesized that the four hydroxycinnamic acid derivatives increase 2DG uptake mediated by PI3K-dependent GLUT4 translocation, whereas chlorogenic acid and cinnamic acid increase 2DG uptake via PPARγ-mediated GLUT 4 translocation (Figure 8). They also significantly reduce the expressions of fatty acid synthase, a regulatory enzyme 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 secondary complications in diabetes. A reduction in fatty acid and cholesterol synthesis might reduce insulin resistance as well as the chance of developing type 2 diabetes.

Theoretical studies indicate that all six phytochemicals have good oral bioavailability,[25−27] so they could be good drug candidates. Other than chlorogenic acid, the other five hydroxycinnamic acid derivatives may absorb through passive diffusion, which might be because of their size.

Administration of antidiabetic herbs with oral hypoglycemic drugs for the treatment of diabetes may pose a potential drug−herb interaction that may have beneficial or adverse effects. It is generally believed that the use of herbs with medicine enhances the effect of the latter and reduces its adverse effects. The results of the present study indicate that the combination of phytochemicals with THZ and metformin could provide an opportunity to reduce the dose of both OHDs, which may help in minimizing their adverse effects as well as achieve enhanced therapeutic effects. At the same time, proper precaution and care should be taken to avoid the severe hypoglycemia that may occur due to combination of these phytochemicals and OHDs. Although these phytochemicals are very safe, research on their long-term usage should be undertaken.

All of the phytochemicals show dose- and time-dependent effects on 2DG uptake. All of the phytochemicals, except cinnamic acid, show synergy with both OHDs, THZ and metformin. These phytochemicals increase glucose uptake either via PI3K or via PPARγ. Caffeic acid, chlorogenic acid, and cinnamic acid reduce the expression of HMG CoA reductase and FAS enzymes. These enzymes are involved in the secondary complications caused by hyperglycemia.[31,32] ADME calculations suggest that these phytochemicals possess drug-like properties. On the basis of the above studies, which suggest synergy between phytochemicals and OHDs, it can be concluded that phytochemicals can partly replace the commercial drugs to achieve the same target, that is, glucose uptake.

■ ASSOCIATED CONTENT

Supporting Information. Additional tables and figure. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +914422574107. Fax: +914422574102. E-mail: mukeshd@iitm.ac.in.

■ ABBREVIATIONS USED

OHDs, oral hypoglycemic drugs; THZ, thiazolidinedione; 2DG, 2-deoxyglucose; GLUT4, glucose transporter 4; PI3K, phosphoinositol-3-kinase; DM, diabetes mellitus; PPAR, peroxisome proliferator activated receptors; DMEM, Dulbecco modified Eagle's medium; FBS, fetal bovine serum; G6PDH, glucose-6-phosphate dehydrogenase; ATP, adenosine triphosphate; DMSO, dimethyl sulfoxide; RNA, ribose nucleic acid; RTPCR, reverse transcriptase polymerase chain reaction; NADPH, reduced nicotinamide adenine dinucleotide phosphate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ORO, Oil Red O; KRPH, Krebs−Ringer−phosphate−Hepes; CI, combination index; TNF, tumor necrosis factor; ADME, absorption, digestion, metabolism, excretion; HMG CoA, 3-hydroxy-3-methylglutaryl-CoA; FAS, fatty acid synthase; HB, hydrogen bond; QPlogBB, predicted brain/blood partition coefficient; QPlogS, predicted aqueous solubility; QPlogPo/w, predicted octanol/water partition coefficient; QPPCaco, predicted apparent Caco-2 cell permeability.

■ REFERENCES

(1) Harris, M.; Zimmet, P. Classification of diabetes mellitus and other categories of glucose intolerance. In *International Textbook of Diabetes Mellitus*; Alberti, K., Zimmet, P., Defronzo, R., Eds.; Wiley: New York, 1997; pp 9−23.

(2) Wild, S.; Roglic, G.; Green, A.; Sicree, R.; King, H. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. *Diabetes Care* **2004**, *27* (5), 1047–1053.

(3) Prabhakar, P. K.; Doble, M. A target based therapeutic approach towards diabetes mellitus using medicinal plants. *Curr. Diabetes Rev.* **2008**, *4* (4), 291–308.

(4) Prabhakar, P. K.; Doble, M. Mechanism of action of medicinal plants towards diabetes mellitus − a review. In *Recent Progress in Medicinal Plants*; Govil, J. N., Singh, V. K., Bhardwaj, R., Eds.; Studium Press: Houston, TX, 2008; Vol. 22, pp 187−210.

(5) Prabhakar, P. K.; Doble, M. Synergistic effect of phytochemicals in combination with hypoglycemic drugs on glucose uptake in myotubes. *Phytomedicine* **2009**, *16* (12), 1119–1126.

(6) Klepacka, J.; Fornal, Ł. Ferulic acid and its position among the phenolic compounds of wheat. *Crit. Rev. Food Sci. Nutr.* **2006**, *46* (8), 639–647.

(7) Croft, K. D. The chemistry and biological effects of flavonoids and phenolic acids. *Ann. N.Y. Acad. Sci.* **1998**, *854*, 435–442.

(8) Liu, R. H. Potential synergy of phytochemicals in cancer prevention: mechanism of action. *J. Nutr.* **2004**, *134* (12 Suppl.), 3479S–3485S.

(9) Scalbert, A.; Manach, C.; Morand, C.; Remesy, C.; Jimenez, L. Dietary polyphenols and the prevention of diseases. *Crit. Rev. Food Sci. Nutr.* **2005**, *45* (4), 287–306.

(10) Lee, E. J.; Kim, S. R.; Kim, J.; Kim, Y. C. Hepatoprotective phenylpropanoids from *Scrophularia buergeriana* roots against CCl_4-induced toxicity: action mechanism and structure-activity relationship. *Planta Med.* **2002**, *68* (5), 407–411.

(11) Wiesner, J.; Mitsch, A.; Wissner, P.; Jomaa, H.; Schlitzer, M. Structure-activity relationships of novel anti-malarial agents. Part 2: Cinnamic acid derivatives. *Bioorg. Med. Chem. Lett.* **2001**, *11* (3), 423–424.

(12) Natella, F.; Nardini, M.; Di Felice, M.; Scaccini, C. Benzoic and cinnamic acid derivatives as antioxidants: structure-activity relation. *J. Agric. Food Chem.* **1999**, *47* (4), 1453–1459.

(13) Lee, H. S. Tyrosinase inhibitors of *Pulsatilla cernua* root-derived materials. *J. Agric. Food Chem.* **2002**, *50* (6), 1400–1403.

(14) Liu, I. M.; Hsu, F. L.; Chen, C. F.; Cheng, J. T. Antihyperglycemic action of isoferulic acid in streptozotocin-induced diabetic rats. *Br. J. Pharmacol.* **2000**, *129* (4), 631–636.

(15) Balasubashini, M. S.; Rukkumani, R.; Viswanathan, P.; Menon, V. P. Ferulic acid alleviates lipid peroxidation in diabetic rats. *Phytother. Res.* **2004**, *18* (4), 310–314.

(16) Hemaiswarya, S.; Doble, M. Synergistic interaction of eugenol with antibiotics against Gram negative bacteria. *Phytomedicine* **2009**, *16* (11), 997–1005.

(17) Hemaiswarya, S.; Doble, M. Mechanistic Studies on Combination of phytochemicals and synthetic drugs as anticancer agents. In *Annals of Traditional Chinese Medicine*; Leung, P.-C., Fong, H., Eds.; World Scientific Publishing: Singapore, 2008; Vol. 3, pp 233−254.

(18) Yamamoto, N.; Sato, T.; Kawasaki, K.; Murosaki, S.; Yamamoto, Y. A nonradioisotope, enzymatic assay for 2-deoxyglucose uptake in L6 skeletal muscle cells cultured in a 96-well microplate. *Anal. Biochem.* **2006**, *351* (1), 139–145.

(19) Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **1983**, *65* (1−2), 55–63.

(20) Gomez, L. A.; Alekseev, A. E.; Aleksandrova, L. A.; Brady, P. A.; Terzic, A. Use of the MTT assay in adult ventricular cardiomyocytes to assess viability: effects of adenosine and potassium on cellular survival. *J. Mol. Cell. Cardiol.* **1997**, *29* (4), 1255–1266.

(21) Laughton, C. Measurement of the specific lipid content of attached cells in microtiter cultures. *Anal. Biochem.* **1986**, *156* (2), 307–314.

(22) Zhao, Z.; Egashira, Y.; Sanada, H. Phenolic antioxidants richly contained in corn bran are slightly bioavailable in rats. *J. Agric. Food Chem.* **2005**, *53* (12), 5030–5035.

(23) Tallarida, R. J. Drug synergism: its detection and applications. *J. Pharmacol. Exp. Ther.* **2001**, *298* (3), 865–872.

(24) Hall, L. R.; Mehlotra, R. K.; Higgins, A. W.; Haxhiu, M. A.; Pearlman, E. An essential role for interleukin-5 and eosinophils in helminth-induced airway hyperresponsiveness. *Infect. Immun.* **1998**, *66* (9), 4425–4430.

(25) Lipinski, C. A.; Lombardo, F.; Dominy, B. W.; Feeney, P. J. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv. Drug Delivery Rev.* **1997**, *23* (1−3), 3–25.

(26) Dossi, K.; Tsirkone, V. G.; Hayes, J. M.; Matoušek, J.; Poučková, P.; Souček, J.; Zadinova, M.; Zographos, S. E.; Leonidas, D. D. Mapping the ribonucleolytic active site of bovine seminal ribonuclease. The binding of pyrimidinyl phosphonucleotide inhibitors. *Eur. J. Med. Chem.* **2009**, *44* (11), 4496–4508.

(27) Duchowicz, P. R.; Talevi, A.; Bellera, C.; Bruno-Blanch, L. E.; Castro, E. A. Application of descriptors based on Lipinski's rules in the QSPR study of aqueous solubilities. *Bioorg. Med. Chem.* **2007**, *15* (11), 3711–3719.

(28) Patel, M. B.; Mishra, S. H. Cell lines in diabetes research: a review. *Pharmacogn. Rev.* **2008**, *2* (4), 188–205.

(29) Sasson, S.; Oron, R.; Cerasi, E. Enzymatic assay of 2-deoxyglucose 6-phosphate for assessing hexose uptake rates in cultured cells. *Anal. Biochem.* **1993**, *215* (2), 309–311.

(30) Sokoloff, L.; Reivich, M.; Kennedy, C.; Rosiers, M. H. D.; Patlak, C. S.; Pettigrew, K. D.; Sakurada, O.; Shinohara, M. The [^{14}C]deoxyglucose method for the measurement of local cerebral glucose utilization: theory, procedure, and normal values in the conscious and anesthetized albino rat. *J. Neurochem.* **1977**, *28*, 897–916.

(31) Zeng, L.; Biernacka, K. M.; Holly, J. M. P.; Jarrett, C.; Morrison, A. A.; Morgan, A.; Winters, Z. E.; Foulstone, E. J.; Shield, J. P.; Perks, C. M. Hyperglycaemia confers resistance to chemotherapy on breast cancer cells: the role of fatty acid synthase. *Endocr.−Relat. Cancer* **2010**, *17* (2), 539–551.

(32) Danesh, F. R.; Kanwar, Y. S. Modulatory effects of HMG-CoA reductase inhibitors in diabetic microangiopathy. *FASEB J.* **2004**, *18* (7), 805–815.