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Mango Extracts and the Mango Component Mangiferin Promote Endothelial Cell Migration

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This study tested the hypothesis that mango extracts contain bioactive molecules capable of modulating endothelial cell migration, an essential step in the formation of new blood vessels or angiogenesis. The formation of new blood vessels is an important therapeutic target for diseases such as limb ischemia, coronary infarction or stroke. We examined the effect of mango peel and flesh extracts as well as the individual polyphenolic molecules, mangiferin and quercetin, on bovine aortic cell migration using a modified Boyden chamber assay. Our results show that mangiferin, and extracts rich in mangiferin, increase endothelial cell migration. The dose−effect relationship for various extracts further suggests that this action of mangiferin is modulated by other components present in the extracts. The promigratory effect of mango extracts or mangiferin was unrelated to an effect on cell proliferation, and did not involve a change in the production of matrix metalloprotease-2 or -9 by the endothelial cells. Taken together, these results suggest that mangiferin present in mango extracts may have health promoting effects in diseases related to the impaired formation of new blood vessels.

KEYWORDS: Angiogenesis; quercetin; mango peel; mango flesh; Boyden chamber

INTRODUCTION

Vascular diseases represent a prominent cause of mortality in Western societies. Whether occlusions affect the coronary or peripheral circulation, neovascularization of the ischemic tissue is an important therapeutic target. Despite intense research and sophisticated approaches including molecular, gene or stem cell-based therapies, there is a great need for effective therapeutic angiogenesis options (*1*). Endothelial cell migration is a critical component of angiogenesis, the process by which new capillaries are formed from preexisting blood vessels. Angiogenesis is a multistep process requiring the degradation of the basement membrane, endothelial cell migration, capillary tube formation, and endothelial cell proliferation. Angiogenesis is required for sustained tumor growth and is prominent in diseases such as rheumatoid arthritis, diabetic retinopathy, and psoriasis. In these pathologies, inhibiting the growth of new vessels presents great clinical interest. On the other hand, inducing or stimulating angiogenesis is desirable in coronary infarction, limb ischemia, stroke, tissue engineering or wound healing.

Many phytochemicals are known to prevent the development and progression of chronic disease. The literature on bioactive molecules from fruits and vegetables from temperate climates is abundant, and recent focus on tropical fruits such as mangos
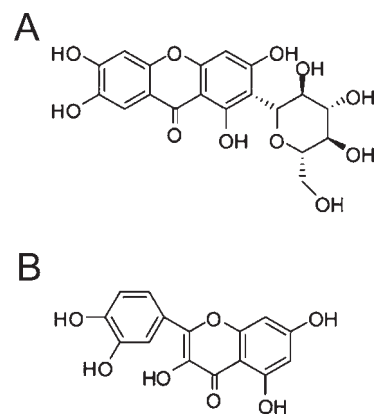

Figure 1. Chemical structures of mangiferin (**A**) and quercetin (**B**).

shows that the flesh and peel of these fruits are also rich in bioactives (*2*, *3*). Mango extracts have been found to possess beneficial effects including anti-inflammatory and antimicrobial activities (*4*, *5*). Mangos contain flavonoids including quercetin and glycosylated xanthones such as mangiferin (**Figure 1**). Quercetin has been shown to possess antioxidant, antimicrobial, antitumor, antihypertensive, antiatherosclerosis and anti-inflammatory properties (reviewed in ref *6*). Quercetin impacts endothelial cell function by inhibiting endothelin-1 effects (*7*), acting as a

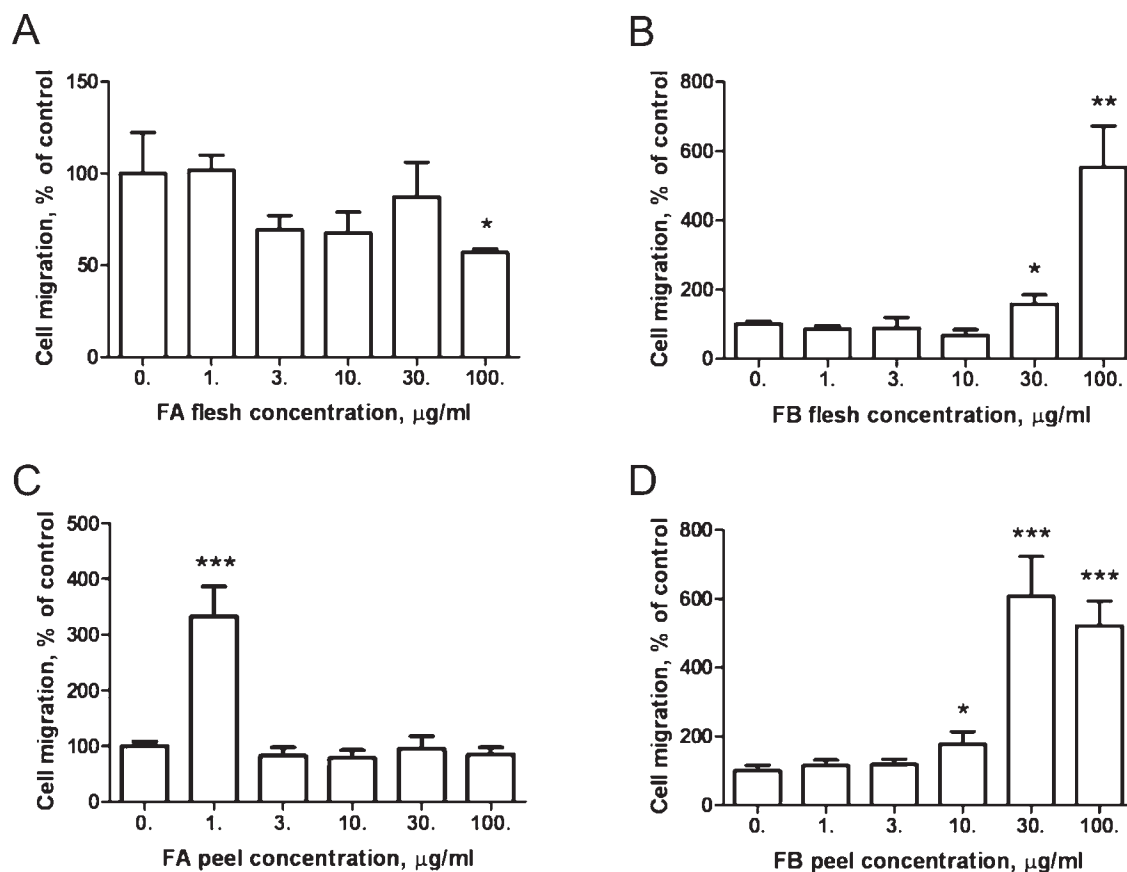*Corresponding author. Tel: +61-7-33651374. Fax +61-7-3365-1688. E-mail m.parat@uq.edu.au.

Figure 2. Effect of mango fractions on bovine aortic endothelial cell migration. Cells in the presence of (**A**) FA flesh, (**B**) FB flesh, (**C**) FA peel, or (**D**) FB peel at the indicated concentrations were allowed to migrate for 4 h across collagen-coated membranes. Nonmigrated cells on the upper surface of the membrane were scraped. Migrated cells on the lower surface of the filter were fixed, stained and counted in three wells per condition, in three separate experiments. Error bars represent standard error of the mean. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

phosphodiesterase type 5 inhibitor (*8*), and preventing endothelial cell migration (*9−11*). Mangiferin has been described as having antioxidant, immunomodulatory, anti-inflammatory and antiviral activities (reviewed in ref *12*). Interestingly, mangiferin also exhibits antidiabetic properties (*13*), and diabetes is a disease context in which increasing angiogenesis is of high interest.

We hypothesized that mango extracts contain bioactives capable of modulating angiogenesis. We tested the effect of mango peel and flesh extracts on bovine aortic cell migration using a modified Boyden chamber assay, in which the cells are induced to migrate through the 8 μm pores of a collagen-coated polycarbonate filter and the migrated cells are counted. This is the most commonly used model/assay to quantitatively assess endothelial cell migration (*14*). We further tested the effect of individual polyphenolic molecules mangiferin and quercetin.

MATERIALS AND METHODS

Materials. Unless otherwise specified, all reagents were purchased from Sigma Aldrich (Sydney, Australia).

Preparation of Mango Extracts. The peel and flesh of ripe mangos (*Mangifera indica* L. cultivar R2E2) were dried separately by lyophilization. A single layer of ca. 1 cm mango cubes was placed in a plastic container and frozen overnight at −18 °C. The frozen samples were placed in the drum manifold of a Virtis BT6K freeze-dryer. The condenser temperature was −82 °C, and a vacuum of 130 μB was applied. The samples were dry after 48 h and were removed for further use. A typical R2E2 mango weighing 500 g provided around 50 g of dried flesh and 10 g of dried peel. Freeze-dried mango flesh (∼25 g) or peel (∼10 g) was placed in a Soxhlet thimble and extracted sequentially under reflux for 6 h with each of the following solvents, hexane (200 mL), ethyl acetate (200 mL)

and water (200 mL). The ethyl acetate and water extracts were combined and acidified to pH 1.5. The acidic solution was further extracted with ethyl acetate (3 × 100 mL), the solvent removed under reduced pressure and the extract stored under nitrogen at −18 °C. The Soxhlet extracts were further fractionated as follows. Dried extract was dissolved in 5% w/v aqueous Na_2CO_3, pH 8 and extracted with ethyl acetate (3 × 100 mL). The ethyl acetate phase was evaporated under reduced pressure to give fraction A. The remaining aqueous phase was acidified to pH 1.5 with 2 M HCl and extracted with ethyl acetate (3 × 100 mL). Ethyl acetate was removed under reduced pressure yielding fraction B (Wilkinson et al., submitted for publication). This method results in fractions A containing phenolic esters and flavonol glycosides while fractions B are enriched in the phenolic acids (*15*). All fractions were stored under nitrogen at −18 °C. From 25 g of freeze-dried flesh ∼100 mg of fraction A (FA flesh) and ∼150 mg of fraction B (FB flesh) were obtained. From 10 g of freeze-dried peel, ∼200 mg of fraction A (FA peel) and ∼100 mg of fraction B (FB peel) were obtained.

Estimation of the Total Phenolic Content of Mango Extracts. The phenolic content of the fractions was determined using the Folin−Ciocalteu assay with gallic acid as a standard (*16*). Results are expressed as mg gallic acid equivalent (GAE) per g of dry weight (DW) of each extract (*17*).

Mangiferin Determination in Mango Extracts by HPLC−MS−MS Analysis. Mango extracts prepared in 10% v/v acetonitrile in water were automatically injected into an Agilent binary HPLC system consisting of an Agilent 1100 LC pump and an Agilent 1100 well plate autosampler and a Synergi 2 μm Fusion-RP 100A C18 (20 × 2.0 mm) HPLC column (Phenomenex, Torrance, CA). The mobile phase consisted of (A) water and (B) acetonitrile, and isocratic elution was performed using a composition of 10% B with a flow rate of 200 μL/min. Standards containing mangiferin (Sigma-Aldrich, batch number 084K1572) concentrations of 2−20 μM in water were injected for calibration. An API

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3000 tandem mass spectrometer equipped with a turbo ion spray interface and supported by Analyst 1.5 software (Applied Biosystems, Foster city, CA) was used to detect the separated compounds and to process all data. A specific transition of molecular ion → fragment was monitored as the detector response in multiple reaction monitoring mode. The fragmentations of 421 *m/z* → 301 *m/z* were monitored to detect mangiferin. The following are the parameters optimized for mangiferin to obtain the highest possible sensitivity: ion spray voltage of −4500 V, entrance potential of −10 V, orifice/declustering potential of −76 V, ring/focusing potential of −350 V, collision energy of −36 V and collision exit potential of −9 V. Curtain gas was set at 12, and nebulizer gas and the collision gas flows were maintained at 8 (in arbitrary units used in the instrument). The temperature of the ion spray was maintained at 400 °C, and a dwell time of 150 ms was used. The resolution of both Q1 and Q3 was 1 amu. Results are expressed as % w/w (or g per 100 g extract).

Cell Culture. Bovine aortic endothelial cells were grown in Dulbecco's modified Eagle's medium and F12 medium supplemented with 5% fetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin, in a humidified atmosphere containing 5% CO2.

Cell Migration Model/Assay. A modified Boyden chamber assay was used to quantify endothelial cell migration (*18*). The chamber was prepared with serum-free medium in the lower well. Polycarbonate membranes with 8 μm pores precoated with rat tail collagen type 1 (100 μg/mL in 0.2 N acetic acid) were used to separate the chambers. Cell suspensions (300,000 cells/mL serum-free medium) were added with mango extracts, mangiferin or quercetin at the indicated concentrations. The final dimethyl sulfoxide concentration was 0.1% for all cell suspensions. After pH equilibration of the chamber in a 5% CO$_2$-containing atmosphere, 50 μL of cell suspension was added to the upper wells. Migration was allowed to proceed for 4 h. The membrane was washed in phosphate-buffered saline (PBS), and the cells remaining on the upper face of the membrane were scraped. The membrane was then fixed in 10% buffered formalin. The membrane was stained with hematoxylin overnight, rinsed with water and mounted using Permount mounting medium. Cells on the lower membrane face were counted microscopically. Experiments were repeated at least three times with each sample run in triplicate. Results are expressed as a percent of the migration of the control cells.

Determination of Cell Viability. Cell viability was determined by mitochondrial function using 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay. Bovine aortic endothelial cells were grown in 96 well plates for 24 h until ∼90% confluent. Cells were incubated with MTT-containing medium (final concentration 0.5 mg/mL) at 37 °C for 2 h. The medium was removed and replaced with DMSO (100 μL per well). Solubilization of the formazan crystals was carried out at room temperature for 20 min and the absorbance read at 570 nm. Results are expressed as a percent of the absorbance of the control cells.

Gelatin Zymography. To prepare conditioned medium, cells were rinsed twice with serum-free medium and incubated with serum-free medium in the presence of mango extracts or individual components for 24 h. The medium was collected, centrifuged for 5 min at (10000*g*) and kept at −18 °C until analysis. Equal amounts of protein from conditioned media were loaded onto a 10% w/v polyacrylamide gel with 1 mg/mL gelatin. After SDS−PAGE separation, gels were incubated in a solution of 50 mM Tris, 5 mM CaCl$_2$, 2.5% Triton X100 to remove the SDS and to help the renaturation of proteases and inhibitors. The gels were then incubated in 50 mM Tris-HCl (pH 7.5) containing 5 mM CaCl$_2$ for 18 h. Gels were stained with 0.25% Coomassie Blue R-250 in 45% methanol, 10% acetic acid, and destained in methanol:water:acetic acid (2.5:6.5:1).

Statistical Analysis. Results are expressed as mean ± standard error of the mean. Differences between treatment groups were analyzed using a *t* test or a one-way analysis of variance where appropriate. *P* < 0.05 was considered significant.

RESULTS AND DISCUSSION

Phenolic Content Analysis of the Mango Extracts. Each extract was estimated for total phenolic content. FA peel (1226 mg GAE/g DW) and FA flesh (1082 mg GAE/g DW) fractions were found, as expected based on the extraction method, to be richer in phenolics than FB peel (389 mg GAE/g DW) and FB flesh (265 mg GAE/g DW).
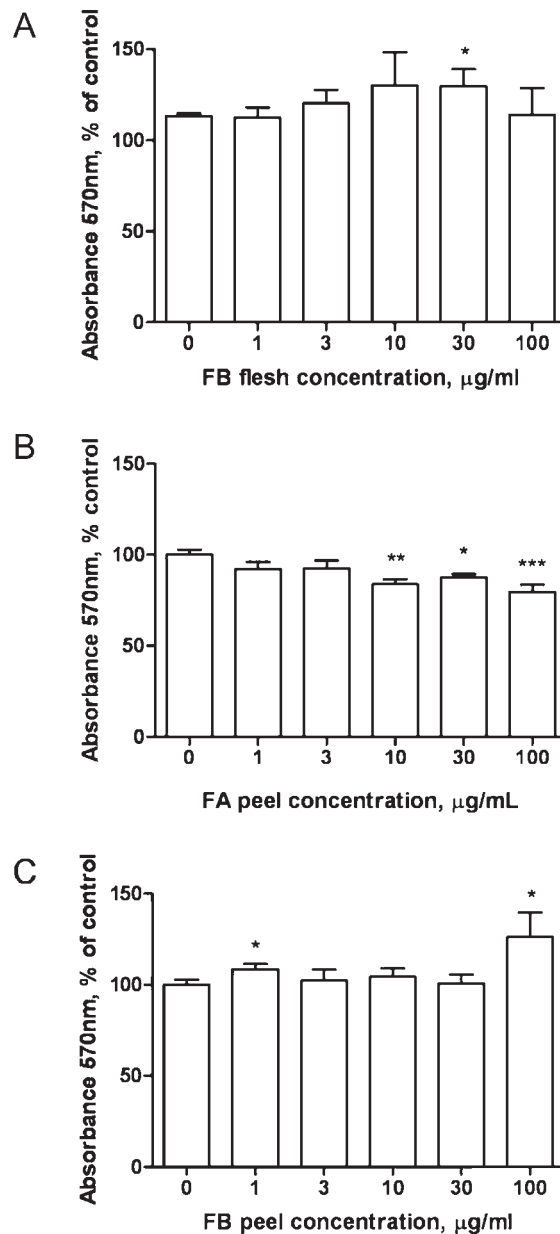

Figure 3. Effect of mango fractions on bovine aortic endothelial cell viability. Cells were exposed to (**A**) FB flesh, (**B**) FA peel, or (**C**) FB peel at the indicated concentrations for 4 h. Cells were incubated with MTT-containing medium for 2 h. The medium was removed and replaced with DMSO and the absorbance read at 570 nm. Error bars represent standard error of the mean. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Effect of the Extracts on Endothelial Cell Migration. The effects of mango peel and flesh extracts on cell migration were measured by adding the different extracts to the cell suspension for the 4 h of migration. Of the four extracts that were tested, three caused an activation of the migration (**Figure 2**). FB flesh and FB peel extracts significantly increased endothelial cell migration in an apparent dose-dependent fashion, the most significant effect being observed at 100 μg/mL for FB flesh and at 30 or 100 μg/mL for FB peel. The FA peel extract activated cell migration at a much lower dose (1 μg/mL), and interestingly, doses higher than 1 μg/mL failed to increase endothelial cell migration. This result was consistently observed in 3 separate experiments. In contrast, the FA flesh extract did not activate endothelial cell migration at any of the doses that we tested. On the contrary, the highest dose (100 μg/mL) decreased cell migration. Because
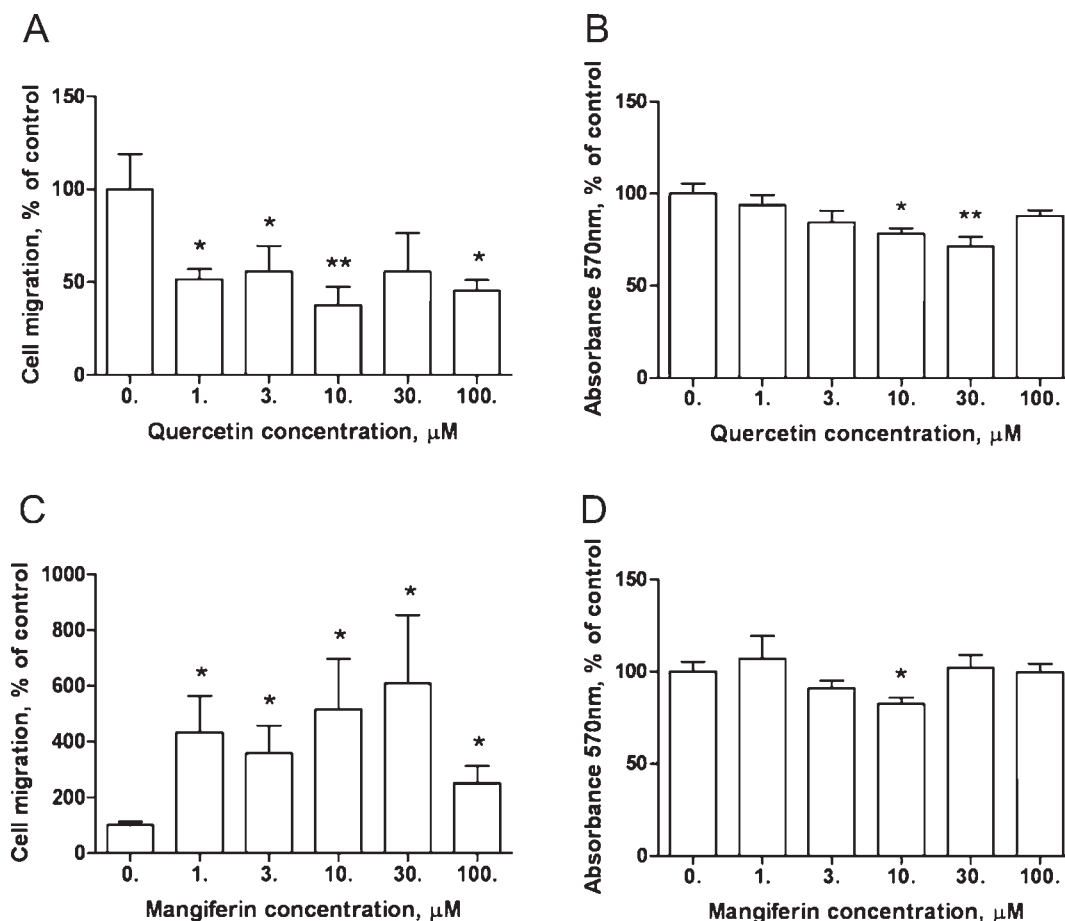
Figure 4. Effect of mango components quercetin and mangiferin on endothelial cell migration and viability. Cell migration in the presence of quercetin (**A**) or mangiferin (**C**) was measured as in **Figure 2**. Cell viability in the presence of quercetin (**B**) or mangiferin (**D**) was measured as in **Figure 3**. Error bars represent standard error of the mean. *, $p < 0.05$; **, $p < 0.01$.

the determination of endothelial cell migration relies on the count of cells reaching the lower surface of the filter on which they are placed, we determined whether the apparent increase in cell migration could in fact be due to an increase in cell proliferation over the duration of the assay. Bovine aortic endothelial cells were exposed to the three extracts that induced increased migration, and cell viability was determined using the MTT assay. Our results show that the increase in migrated cells was not attributable to an increase in cell numbers due to activation of proliferation by the extracts (**Figure 3**). The FB peel extract at the highest dose (100 µg/mL) caused a slight increase in cell viability (**Figure 3C**) but not enough to account for the 5-fold increase in migrated cell numbers that were seen in the Boyden chamber assay. Therefore, our results show that the FB flesh, FA peel and FB peel mango extracts activate endothelial cell migration.

Effect of Isolated Mango Bioactives on Endothelial Cell Migration. We next determined the effect of two individual molecules known to be present in mangos, namely, the flavonoid quercetin and the glycosyl xanthone mangiferin, on endothelial cell migration. Quercetin induced a decrease in migration at all doses tested (from 1 to 100 µM) (**Figure 4A**). Quercetin also induced a slight decrease in cell viability, as determined by the MTT assay (**Figure 4B**). This is in agreement with abundant literature reporting that quercetin has antiangiogenic properties. Quercetin inhibited viability and migration of bovine aortic endothelial cells with an IC_{50} of 15 and 19 µM respectively, and limited *in vitro* tube formation with an IC_{50} of 37 µM (*10*). These *in vitro* antiangiogenic properties have subsequently been confirmed

using choroids-retinal endothelial cells (*9*) and microvascular and umbilical endothelial cells (*11*). Quercetin was further shown to inhibit angiogenesis in the chicken chorioallantoic membrane assay (*11*).

In contrast, mangiferin increased cell migration at all concentrations tested (**Figure 4C**). This increase in the number of migrated cells was not due to an increase in cell proliferation (**Figure 4D**). Furthermore, the increase in cell migration elicited by mangiferin was greater (up to 600%) than the decrease induced by quercetin (∼50%) for a similar dose range. These experiments show for the first time that mangiferin activates endothelial cell migration, highlighting the proangiogenic potential of this mango component. The concentrations of mangiferin showing promigratory activity ranged from 1 to 30 µM. This is compatible with plasma concentrations obtained in animal models following oral administration of mangiferin-rich extracts and traditional oriental medicines known to be rich in mangiferin (*19*, *20*). In rats orally administered an aqueous mangiferin solution at a dose of 120 mg/kg, the plasma concentration of mangiferin was found to be around 19 µM (*21*). This indicates that endothelial cells can be exposed *in vivo* to concentrations of mangiferin that promote angiogenesis following administration of extracts or supplements. This is in accordance with the angiogenesis-promoting potential of mangiferin suggested in one other study: In a rat model of periodontal disease, histology detected a significant increase in the number of blood vessels in the area of the experimental injury when rats were orally given 100 mg/kg per day mangiferin but not in control, saline-treated animals. The experiment was carried out over a period of 7 days (*22*). The LD_{50} of mangiferin has been

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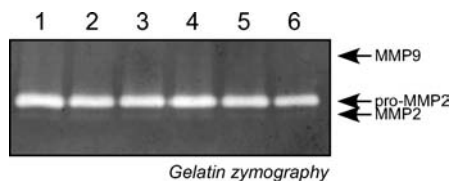*J. Agric. Food Chem.,* Vol. 58, No. 8, 2010 **5185**

Figure 5. Effect of mango fractions and components on the production of matrix metalloproteases. The 24 h conditioned medium of bovine aortic endothelial cells exposed to DMSO alone (lane 1) or to 100 μg/mL of FB flesh (lane 2), 30 μg/mL of FB peel (lane 3), 1 μg/mL of FA peel (lane 4) 10 μM of quercetin (lane 5) or 30 μM of mangiferin (lane 6) was analyzed by gelatin zymography. The distances run by recombinant matrix metalloprotease 2 (MMP2) and recombinant matrix metalloprotease 9 (MMP9) are indicated by arrows.

evaluated in mice and was reported to be 400 mg/kg body weight after intraperitoneal injection (*23*).

Mangiferin Content of the Extracts. The capacity of the mango extracts to promote endothelial cell migration does not correspond to their total phenolic content. We hypothesized that mangiferin might be, in part, responsible for the promigratory activity of the mango extracts. We therefore evaluated the concentration of mangiferin present in the four extracts using HPLC−MS−MS analysis. Mangiferin was below the detection limit in FA flesh, and was found to be 0.34% w/w, 0.95% w/w and 0.60% w/w in FB peel, FA peel and FB flesh respectively. It is worth noting that the fraction-A flesh (FA flesh) did not increase endothelial cell migration at the doses tested, and also had undetectable amounts of mangiferin. Therefore, the mango fractions that are rich in mangiferin are also the ones that exhibit promigratory properties.

Interestingly, the doses of the three other extracts that induced maximal increase in migration corresponded to concentrations of mangiferin between 0.02 μM and 1.42 μM. Such observations are compatible with the hypothesis that mangiferin may be the bioactive responsible for the promigratory activity of the extracts, but they also should be considered in the context of the chemical complexity of the extracts. Extracts contain multiple bioactives, and these may positively or negatively influence each other's effect on endothelial cell migration. The biphasic effect seen with FA peel (which was detected in three separate experiments) indicates that other compounds with an antagonistic effect on endothelial cell migration may be present in the extract. In contrast, the 5- to 6-fold increase in migration induced by 30 μg/mL of FB peel or 100 μg/mL of FB flesh, corresponding to 0.2 μM and 1.42 μM of mangiferin respectively, are unlikely to be explained by the sole presence of mangiferin but most likely by the additive or synergistic effect of other phytochemicals present in the B fractions.

Effect of Mango Extracts or Mangiferin on Matrix Metalloproteases. Matrix metalloproteases (MMPs) play a key role in angiogenesis by allowing the digestion of the basement membrane, but also by mobilizing proangiogenic proteins from the stroma, or cleaving physiological macromolecules to generate angiogenic regulators (*24*). To determine whether mango fractions or molecular components could increase cell migration via an increase in MMP production, we analyzed the conditioned medium of endothelial cells exposed to FA peel, FB peel, FB flesh or mangiferin at the dose that increased endothelial cell migration the most. We also included quercetin at the dose that inhibited cell migration the most (**Figure 5**). Analysis of the conditioned medium was performed using gelatin zymography. MMP9 was undetectable in control or treated samples. Abundant levels of pro-MMP2 were detected, with only around 5% of the total

MMP2 present in the state of active, cleaved enzyme. No change in MM2 was detected when the endothelial cells were exposed to mango fractions or mango purified molecules. A mechanism for the antiangiogenic action of quercetin has been suggested to be the decreased expression and activity of matrix metalloprotease-2 (*11*). In the latter study, doses of quercetin of 25−100 μM were shown to exert an inhibitory effect. Our results did not show an effect of 48 h 10 μM quercetin on MMP-2 production, but did show a decrease in EC migration when cells were exposed to quercetin for 4 h, indicating that the effect on migration at this lower dose is independent from MMP2.

This study has shown that mangiferin and mango fruit extracts that contain mangiferin promote endothelial cell migration, a key process in angiogenesis. Other components in mango extracts may also contribute to the activity and/or modulate it. Together with demonstrated bioactivities such as PPAR activation and inhibition of cancer cell proliferation (ref *25* and Wilkinson et al., submitted), these results demonstrate the diverse bioactivities present within mango fruit.

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