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Extract of *Aquilaria crassna* leaves and mangiferin are vasodilators while showing no cytotoxicity

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ABSTRACT

The leaves of *Aquilaria* spp. promote “physiological balance”, and are “cardiotonic and provide blood nourishment”. In Asia, these leaves are increasingly consumed as tea and claimed to provide benefits to cardiovascular function, albeit without any scientific proof. Therefore, this study sought to evaluate the action of *Aquilaria crassna* leaf aqueous extract (AE) on vascular function and vascular smooth muscle cytotoxicity. AE and a main constituent, mangiferin were investigated for their vasorelaxation of rat mesenteric arteries and aortae *in vitro*. Acute cytotoxicity of AE (0.1–1000 µg/ml) and mangiferin (0.1–100 µM) on rat enzymatically isolated vascular smooth muscle cells was assayed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide. AE dilated rat mesenteric arteries (EC₅₀~107 µg/ml, E_{max}~95%) more than aorta (EC₅₀~265 µg/ml, E_{max}~76%, *p* < 0.05).

AE-induced vasodilation in mesenteric artery was reduced by endothelial removal (EC₅₀~202 µg/ml, *p* < 0.05), incubation with endothelial nitric oxide synthase (eNOS) (100 µM, L-NAME) (EC₅₀~309 µg/ml, *p* < 0.05), and partly reduced by L-type Ca²⁺ channel blockade at higher concentrations. Likewise, mangiferin (1–100 µM) dilated the mesenteric artery more potently than the aorta. However, its maximum relaxation was less than with AE (41% in the mesenteric artery and <10% in the aorta). Isolated vascular smooth muscle cells incubated in AE or mangiferin for 1 h showed no cytotoxicity. Thus, AE is a vasorelaxant while being free of acute cytotoxicity towards vascular smooth muscle, thus potentially ameliorating human vascular dysfunction.

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1. Introduction

Aquilaria crassna Pierre ex Lecomte is a medicinal herb from *Aquilaria* species of Thymelaeaceae family. *Aquilaria* spp. trees, also known as agarwood, are mainly found in Southeast Asia. Their fragrant resinous wood has long been used in Ayurvedic and traditional medicine for treatment of various illness such as joint pain, inflammatory-related disorder, diarrhea, and also used as a

sedative, stimulant and cardio-protectant.¹ In Thailand, agarwood is the main ingredient in the herbal remedy called “Yahom” used for treatment of fainting and abdominal discomfort.² Several reports describe biological activities in the resin derived from heartwood and in the leaves of *Aquilaria* spp.¹ Anti-oxidant,^{3,4} anti-pyretic,³ anti-diabetic,⁵ anti-microbial⁶ and hepatoprotective⁷ properties of the extract from leaves of *Aquilaria* spp. including *A. crassna* have been recently described.

Some of these effects are replicated by a constituent of *A. crassna*, mangiferin, a xanthone also found in variety of plant species.^{5,8–10} Such pharmacological properties of mangiferin,^{8,11–13} include anti-oxidation,¹⁴ anti-inflammation,¹⁵ anti-cancer,^{16,17} and anti-diabetic.¹⁸ Its protective effects in various organs including heart,¹⁹ brain,²⁰ kidney,¹⁹ and liver²¹ are well

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established. Clearly, therapeutic and preventive properties of mangiferin are extensive, thus making plants containing mangiferin potential sources of medication. While most mangiferin effects relate to its anti-oxidant and anti-inflammatory activities, its direct action on the vascular system has not been fully characterized.

In Asia, *A. crassna* leaves are consumed as herbal tea and are vaguely claimed to promote physiological balance and more specifically improve cardiovascular function, but there appears to be little direct evidence for this assertion. Therefore, present study aimed to investigate the vascular action of aqueous extract of *A. crassna* and its constituent, mangiferin on rat isolated aorta and mesenteric artery. To assess their safety, cytotoxicity of the extract and mangiferin was tested on rat isolated vascular smooth muscle cells (VSMCs).

2. Materials and methods

2.1. Plant collection and extraction

Young leaves (1–3 leaves from the top) of *A. crassna* were collected from Phitsanulok Province, Thailand. The plants were identified by Dr. Pranee Nanggam, Department of Biology, Faculty of Science, Naresuan University. The specimen voucher (Collection number: Wongwad001) was kept at Department of Biology Herbarium, Faculty of Science, Naresuan University. The leaves were dried at 100 °C for 3 h and ground. Then, the powdered sample was infused in water maintained at 95–100 °C (1:10 w/v) for 30 min and the supernatant filtered and lyophilized to obtain dried aqueous extract of *A. crassna* leaves (AE) with a 19.8 ± 2.2% yield. HPLC revealed iriflophenone 3, 5-C-β-D-digluconide (13.5%), iriflophenone 3-C-β-D-glucoside (4.6%), mangiferin (9.5%), genkwain 5-O-β-primevosiide (0.9%) (Fig. 1).

2.2. Animals and vessel preparation

Male Sprague Dawley rats (200–250 g) were purchased from the National Laboratory Animal Centre, Mahidol University, Nakhorn Pathom, Salaya, Thailand, kept under 12 h each light:dark

cycle, at 22 ± 1 °C and allowed free access to standard food and water. All experimental protocols were approved by Naresuan University Animal Care and Use Committee (NUACUC, Naresuan University, Phitsanulok, Thailand, approval reference: NU-AE570619). Rats were anesthetized with 50 mg/kg sodium pentobarbital (IP) and supplemented as needed. Afterwards, thoracic aorta, primary branch of mesenteric artery and tail artery were excised and cleaned of connective tissue. Aorta and mesenteric artery were cut into rings 2–5 mm in length and suspended in water-jacketed glass baths via stainless steel loops connected to tension transducers. Rings were bathed in Krebs' solution (mM): NaCl, 122; KCl, 5; [N-(2-hydroxyethyl) piperazine N'-(2-ethanesulfonic acid)] HEPES, 10; KH₂PO₄, 0.5; NaH₂PO₄, 0.5; MgCl₂, 1; glucose, 11; and CaCl₂, 1.8 (pH 7.3), at 37 °C and bubbled with air. The vessel rings were allowed to equilibrate for 1 h at a resting tension of 1 g.^{22,23} In some rings, endothelium was mechanically denuded via a luminal wire. Following equilibration, each ring was tested for contractile viability by applying 10⁻⁵ M phenylephrine (PE), then endothelium function tested using 10⁻⁵ M acetylcholine (ACh) to induce more than 70% relaxation of the pre-contracted vessels.

2.3. Vascular reactivity study

To investigate the vasodilator action of AE and mangiferin, endothelium-intact aortic and mesenteric arterial rings were pre-contracted with 10⁻⁵ M PE until contraction reached plateau, and then 0.1–1000 µg/ml AE or 0.1–100 µM mangiferin were added cumulatively to obtain concentration-relaxation curves. Similar protocols were applied to endothelium-denuded mesenteric arteries. Solvent (DMSO) controls for mangiferin were also run.

The role of nitric oxide released from the endothelium was tested by pre-application of N^o-nitro-L-arginine methyl ester (100 µM, L-NAME), an inhibitor of endothelial nitric oxide synthase (eNOS) for 30 min, followed by accumulating concentrations of AE extract.

Inhibition of extracellular Ca²⁺ influx by AE was evaluated using protocol as previously described.²² Briefly, endothelium-denuded mesenteric arterial rings were pre-incubated in Ca²⁺-free Krebs'

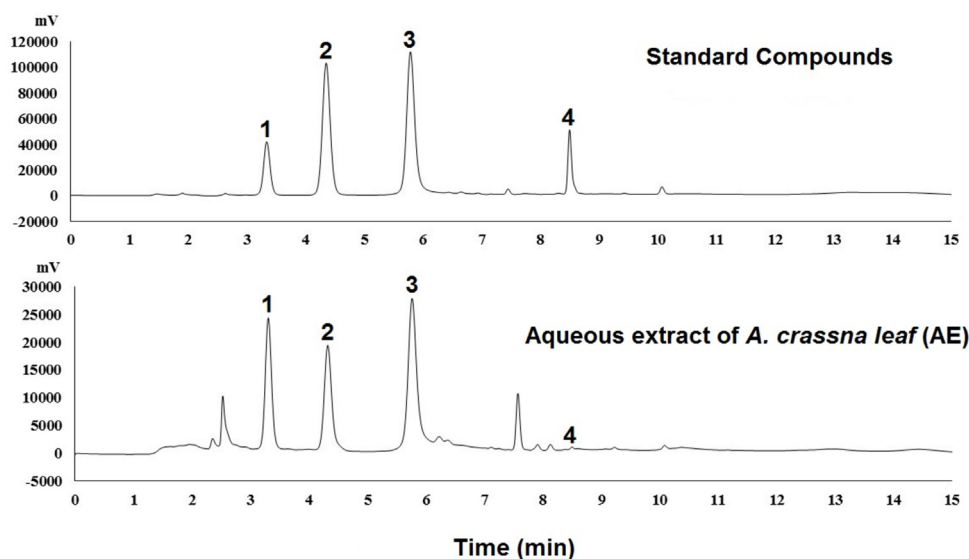


Fig. 1. HPLC fingerprint of aqueous extract of *A. crassna* leaf. The peaks are identified by comparing to reference standards as following: (1) iriflophenone 3, 5-C-β-D-digluconide, (2) iriflophenone 3-C-β-D-glucoside, (3) mangiferin, (4) genkwain 5-O-β-primevosiide. The Phenomenex Luna C18 column (150 mm × 4.6 mm, 5 µm particle size) and a guard column (5 µm Phenomenex C18, 4 mm × 3 mm) were used. Mode of elution was gradient with acetate buffer pH 3.70 and acetonitrile at 1.0 ml/min. The UV detector was set at 310 nm and the injection volume was 20 µl.

solution containing 10 mM ethylene glycol-bis (2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) for 30 min, followed by Ca²⁺-free Krebs' solution containing 80 mM K⁺ for 10 min to open voltage-operated Ca²⁺ channels. CaCl₂ (0.01–10 mM) was then added cumulatively to obtain concentration-contraction responses. This protocol was repeated in the same rings but pre-incubated with AE (either 10, 100 or 1000 µg/ml) in Ca²⁺-free solution (control) for 10 min followed by CaCl₂ added accumulatively.

2.4. Vascular smooth muscle cell (VSMC) isolation and cytotoxicity study

Using rat tail artery, VSMC were isolated as previously described.²⁴ Briefly, tail artery was cut into small strips and opened longitudinally. The strips were immersed in dissociation medium (DM) containing (mM): NaCl, 110; KCl, 5; KH₂PO₄, 0.5; NaH₂PO₄, 0.5; NaHCO₃, 10; HEPES, 10; phenol red, 0.03; taurine, 10; EDTA, 0.5; MgCl₂, 2.0; glucose, 10 and CaCl₂ 0.16 adjusted to pH 7.0 with 1 N NaOH, then incubated for 1 h at 4 °C in DM containing 1 mg/ml papain, 0.04% bovine serum albumin (BSA) and 0.4 mM 1,4-dithiothreitol (DTT) and further incubated at 37 °C for 15 min. 1 mg/ml collagenase was then added and further incubated for 5 min at 37 °C. Tissues were transferred into fresh DM and dispersed by gentle trituration with a glass Pasteur pipette until isolated VSMCs appeared in the bathing solution.

Acute toxicity of AE and mangiferin on VSMCs was investigated by determining cell viability; quantified (n = 5) by cell counting with a hemocytometer using 0.3% trypan blue penetration (Gibco™, Thermo Scientific, USA) and was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. VSMCs were seeded on 96-well plates at 5 × 10⁵ cells/well and treated with AE (0.1–1000 µg/ml), mangiferin (0.1–100 µM) or 0.1% DMSO. After treatment for 1 h at 37 °C, cells were incubated with MTT solution (0.5 mg/ml) for 4 h at 37 °C, then solutions were

aspirated, DMSO:EtOH (1:1) added and incubated on a shaker plate for 15 min in the dark. The absorbance was read at 595 nm by spectrophotometry. VSMCs viability was calculated as percentage of absorbance compared with control. Viability of control cells (no treatment) was considered as 100%.

2.5. Drugs and solution

PE, ACh, papain, collagenase type IA, taurine, BSA, DTT, DMSO, phenol red, MTT, EGTA and HEPES were obtained from Sigma (St. Louis, MO, USA). Mangiferin was a gift from Dr. Uthai Wichai, Department of Chemistry, Naresuan University, Phitsanulok, Thailand and was dissolved in DMSO. MTT was dissolved in PBS (at pH 7.4). Other substances were dissolved in distilled water.

2.6. Statistical analysis

All data were expressed as mean ± standard error of mean (SEM) of *n* animals. The AE extract or mangiferin induced vasorelaxation was calculated as the percentage of the contraction to PE. The EC₅₀ and E_{max} values were determined by fitting the original concentration–response curves using Graph Pad Prism software (version 5.0) and compared using unpaired Student's *t*-test. Concentration–response relationships or multiple comparisons were analyzed using two-way ANOVA followed by Tukey's test. *p* < 0.05 was considered statistically significant.

3. Results and discussion

3.1. AE and mangiferin relaxed mesenteric artery more than aorta

AE produced concentration-dependent relaxation of endothelium-intact (+E) mesenteric arteries (E_{max} = 94.6 ± 2.5%), but less so for the aorta (E_{max} = 76.4 ± 9.2%) (Fig. 2). The corresponding EC₅₀s were 107.3 ± 58.3 µg/ml in mesenteric artery and

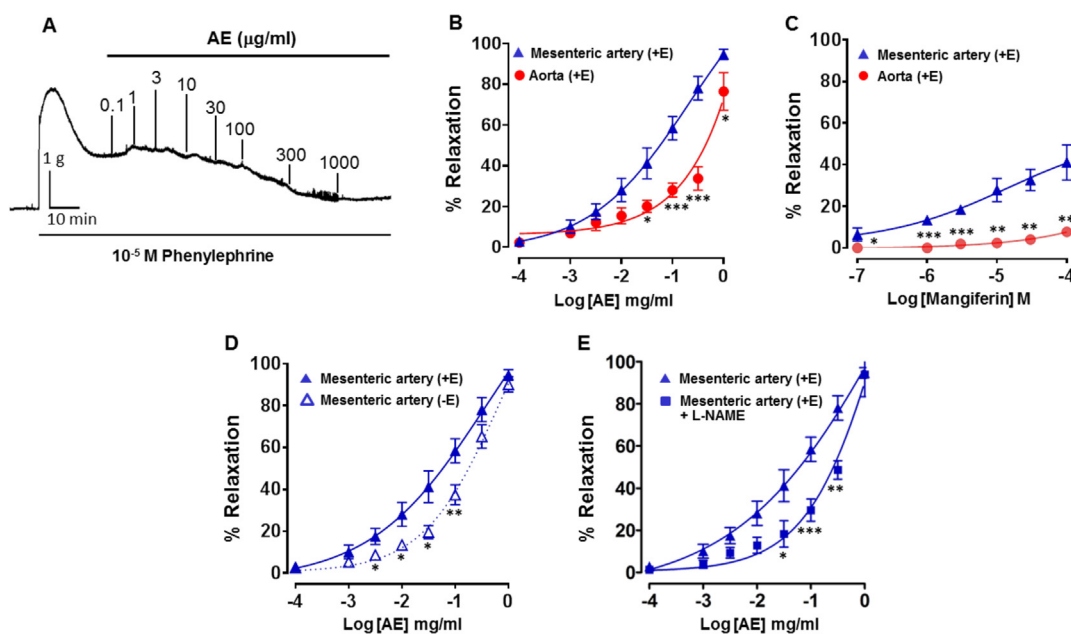
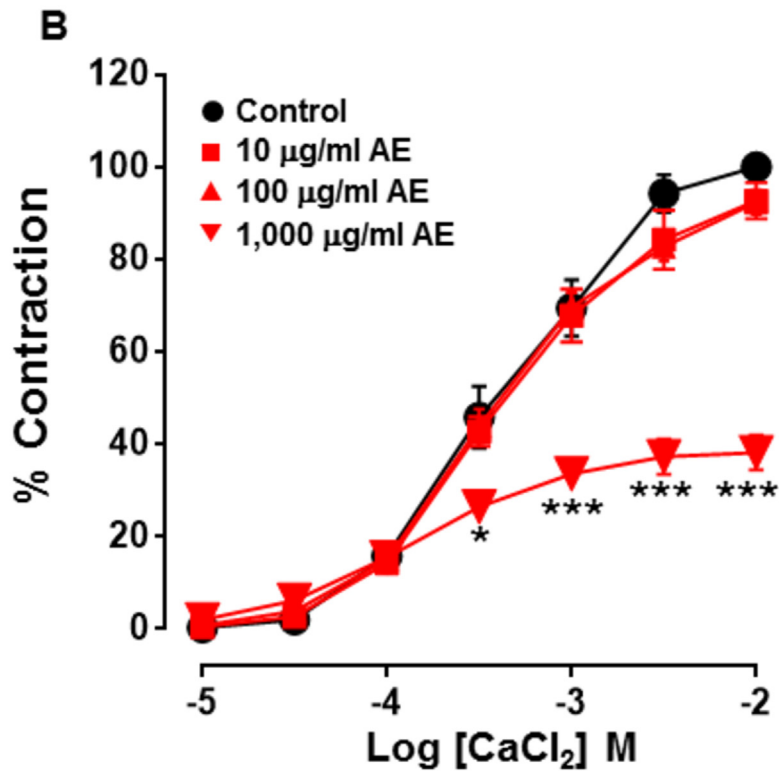
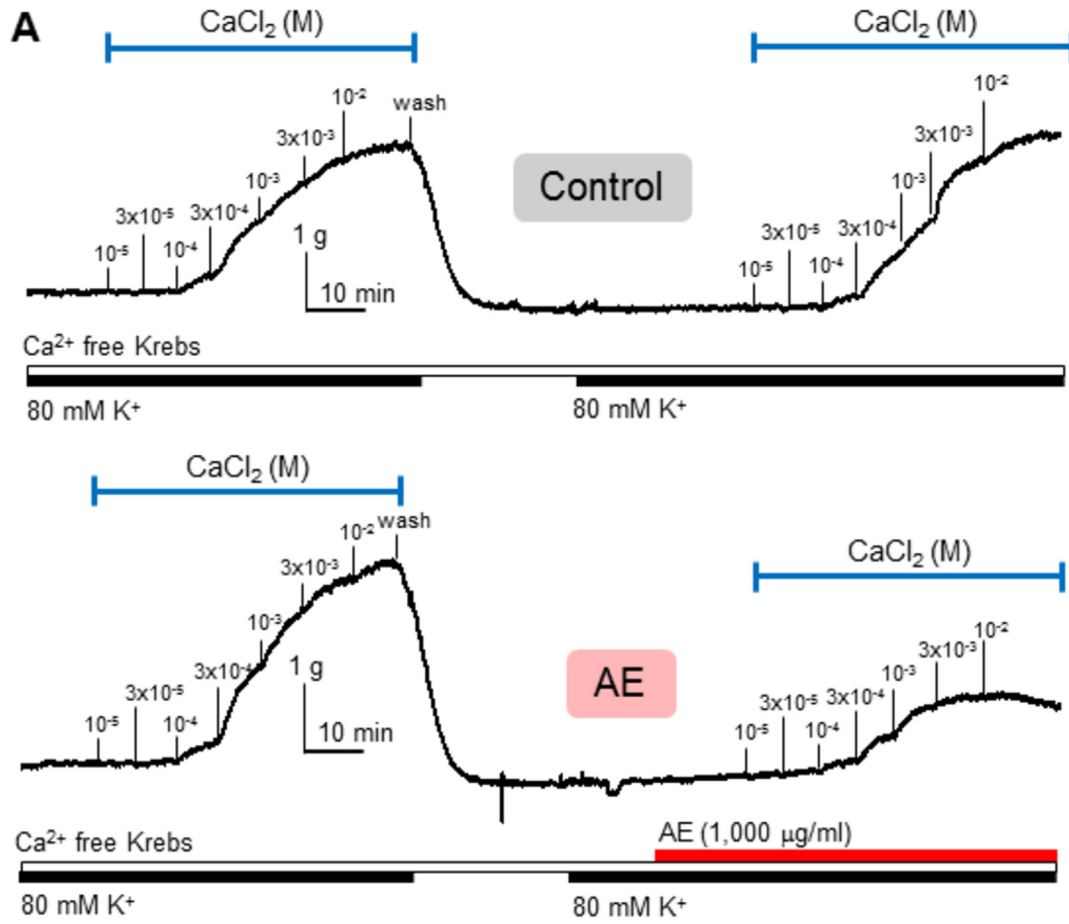


Fig. 2. (A) A representative record showing vasorelaxation by *A. crassna* extract (AE) (0.1–1000 µg/ml) of mesenteric arterial ring pre-contracted with 10⁻⁵ M PE. (B). Concentration-relaxation curves for AE and mangiferin in endothelium-intact (+E) mesenteric artery (n = 10) and (+E) aorta (n = 6). (C) Similar protocols for mangiferin (mesenteric, n = 4; aorta, n = 3). (D) Concentration-relaxation curves for AE in (+E) mesenteric artery compared with endothelium-denuded (-E) mesenteric artery (n = 9). (E) Concentration-relaxation curves for AE in (+E) mesenteric arteries with and without pre-incubation with eNOS inhibitor (L-NAME) (n = 4). Relaxation is expressed as percentage of the contraction induced by PE. All data are expressed as mean ± SEM. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 compared with (+E) mesenteric artery.



202.3 ± 64.1 µg/ml in aorta suggesting some selectivity of AE towards mesenteric arteries. Vasodilation of the mesenteric vasculature contributes substantially to systemic blood pressure and may translate into an anti-hypertensive effect, but needs confirming by *in vivo* and pharmacokinetic studies. There was a similar selectivity for the mesenteric artery over the aorta for a major constituent of AE, mangiferin, albeit with maximal vasorelaxations of only 41% for mesenteric artery and <10% in aorta (Fig. 2C). DMSO had no effect (data not shown). However, mangiferin previously was reported to have no effect on noradrenaline (NA)-induced contraction in resistance mesenteric arteries isolated from normotensive Wistar Kyoto and spontaneously hypertensive rats.²⁵ This may reflect the variant protocols, in particular that these authors pre-incubated mangiferin (0.05 mg/ml) and measured NA-induced contractions. This contrasts with our study, which examines pre-contraction by PE, followed by testing mangiferin relaxations. In agreement with small effect on aorta, mangiferin (100 µM) failed to relax aortic rings pre-contracted with NA.²⁶ Other constituents of AE as shown in Fig. 1 might as well contribute to vasorelaxant action of the extract.

3.2. AE induced endothelium-dependent and -independent vasorelaxation in mesenteric artery

Removal of the endothelium decreased the relaxant effect of AE in mesenteric artery: EC₅₀ increased from 107 ± 58 µg/ml to 202 ± 64 µg/ml, $p < 0.05$ (Fig. 2D) suggesting an action through both endothelium-dependent and -independent pathways directly on VMSC.

To determine the role of the endothelium in the AE-induced relaxation, vessels were pre-incubated with L-NAME (100 µM), an endothelial nitric oxide (NO) synthase (eNOS) inhibitor. This reduced the potency of AE-induced vasorelaxations, (EC₅₀ = 107.3 ± 58.3 µg/ml in control and 309.4 ± 79.7 µg/ml with L-NAME), $p < 0.05$ (Fig. 2E), while E_{max} was unaffected (93.9 ± 10.5% in control and 94.6 ± 2.5% by L-NAME). This suggests that the NO pathway accounts for the entire endothelial component of the AE-induced relaxation.

The remaining AE-induced relaxation in endothelium-denuded (-E) mesenteric arteries implies that AE also acts directly on vascular smooth muscle. A possible mechanism for this might be through interfering with influx of extracellular Ca²⁺. This was tested by elicited Ca²⁺-influx by cell depolarization with 80 mM K⁺ in the presence of increasing Ca²⁺-concentrations (Fig. 3A). This influx occurs through voltage-operated Ca²⁺ channels i.e., L-type Ca²⁺ channels which were blocked by nifedipine.²² Pre-incubation of mesenteric artery with the highest AE concentration (1000 µg/ml) inhibited the Ca²⁺-induced contraction: E_{max} was 38.1 ± 3.7% compared to vessels with AE (100%), $p < 0.001$ (Fig. 3B).

However, lower AE concentrations of AE (10, 100 µg/ml), had little influence on CaCl₂-induced contraction which still eliciting a substantial relaxant action in PE pre-contracted (-E) vessels. This implies that the direct inhibition of contraction is bimodal, one action at lower AE concentration and the other through L-type Ca²⁺-channels only at higher AE concentrations.

The other mechanisms might be activation of the soluble guanylyl cyclase/cyclic guanosine monophosphate pathway and smooth muscle K⁺ channels. Indeed, these mechanisms contribute to mangiferin-induced relaxations in guinea pig isolated trachea.²⁷

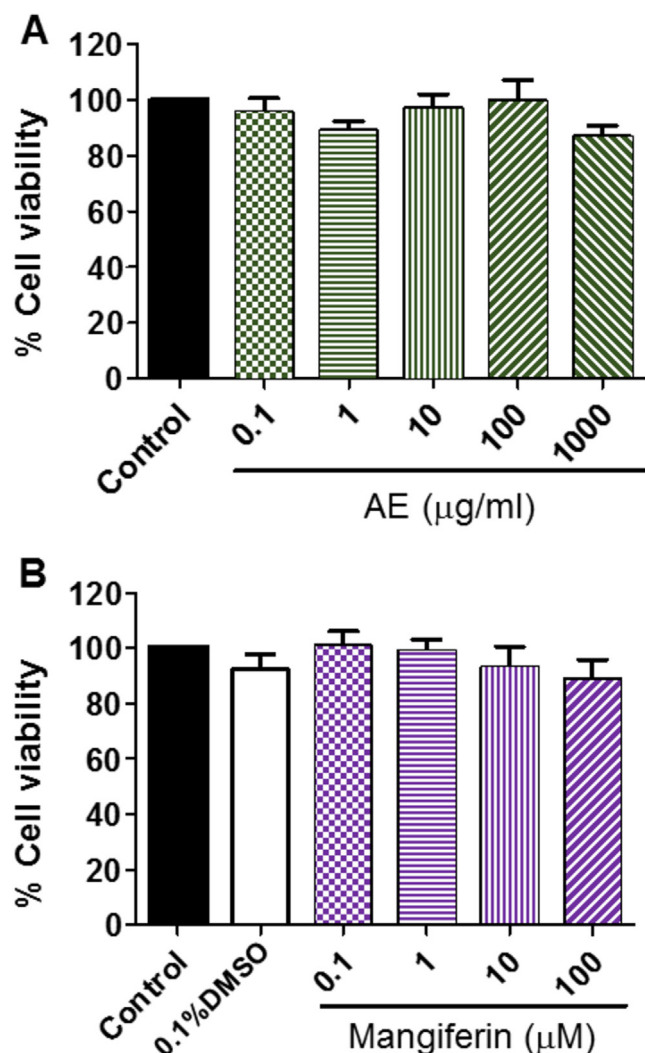


Fig. 4. (A) Cytotoxicity testing of 0.1–1000 µg/ml *A. crassna* extract (AE) or (B) 0.1–100 µM mangiferin for 1 h on rat isolated vascular smooth muscle cells. Cell viability was determined by MTT assay. All data are expressed as mean ± SEM (n = 5).

3.3. Cytotoxicity of AE and mangiferin on VSMCs

Cytotoxicity of AE and mangiferin towards rat isolated VSMCs was assessed by the MTT assay. Incubation of isolated VSMCs with either AE (0.1–1000 µg/ml) or mangiferin (0.1–100 µM) for 1 h had no influence on viability of VSMCs (Fig. 4) demonstrating that neither AE nor mangiferin had acute cytotoxicity that might account for their apparent vasodilator actions.

4. Conclusions

This study demonstrated that AE and a major constituent, mangiferin, induced vasodilation in mesenteric artery, partially via endothelium-dependent NO release, and at higher AE concentrations by directly acting on vascular smooth muscle by blocking L-type voltage-operated Ca²⁺ channels. Neither AE nor mangiferin

Fig. 3. (A) Representative records showing CaCl₂-induced contraction of mesenteric artery in the absence (control) or presence of *A. crassna* extract (AE). Isolated endothelium-denuded mesenteric arterial rings were bathed in Ca²⁺-free high K⁺-solution (80 mM) containing 10 µM EGTA and contraction evoked by cumulative concentration of CaCl₂ was measured. This protocol was then repeated alone (control, n = 4) or in the presence of 10, 100, or 1000 µg/ml AE (n = 7). (B) Concentration-response curves for CaCl₂-induced contraction of rat mesenteric artery in the absence (control) or presence of AE. The CaCl₂-induced contraction was calculated as percentage of the maximum contraction recorded from the first CaCl₂ application and expressed as mean ± SEM. * $p < 0.05$, *** $p < 0.001$ compared with control, 10 and 100 µg/ml AE.

showed any cytotoxicity on VSMCs. Thus this may clinically translate to beneficial effects on vascular function and treatment of cardiovascular diseases including hypertension, but await further characterization of AE and its other constituents.

Conflicts of interest

All authors declare that they have no conflicts of interest.

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