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Moringa Oleifera Lam Leaves Extract and Its Compounds Effect on The Intracellular Calcium Release in Human Aortic Smooth Muscle Cells

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ABSTRACT

Moringa oleifera leaves have been scientifically shown to lower blood pressure, but the mechanism of action on this has not been fully explored. Intracellular calcium release is a fundamental cellular process linked to vascular function and modulation of this process can have significant implications for cardiovascular health. The blocking effects of calcium influx and release by *Moringa oleifera* Lam leaf extract and its compounds on the human aortic smooth muscle cells (HAoSMCs) were studied. The cell viability assay was carried out to determine the concentrations of *M. oleifera* Lam leaf extract and its compounds for the assay. Test compounds (amlodipine, chlorogenic acid, gallic acid, kaempferol, *M. oleifera* leaf extract, quercetin and vicenin-2) were incubated with Quin-2-AM in the presence and absence of extracellular calcium (Ca^{2+}) and with the addition of caffeine and potassium (K^{+}) in HAoSMCs. Following treatments with the test compounds, the influx of extracellular Ca^{2+} into the cytosol of HAoSMCs was significantly inhibited ($p < 0.001$). The test compounds significantly ($p < 0.001$) decreased the concentration of cytosolic Ca^{2+} induced by K^{+} depolarisation. The test compounds also significantly ($p < 0.001$) affected the intracellular Ca^{2+} metabolism, regulated by the caffeine-sensitive storage site in HAoSMCs. No synergistic effect on the decreasing of cytosolic Ca^{2+} level was observed when the test compounds were used in combination with amlodipine. *M. oleifera* Lam leaf extract and its compounds may act as Ca^{2+} channel blockers (CCBs) and there is also a possibility of interactions between the test compounds and amlodipine.

Keywords: amlodipine, cytosolic Ca^{2+}, intracellular Ca^{2+}, K^{+} depolarisation, *Moringa oleifera*

INTRODUCTION

M. oleifera is widely found in the tropical and subtropical regions. It is rich in simple sugars (glucosinolates and isothiocyanates), alkaloids (moringine and moringinine), flavonoids (kaempferol, rhamnetin, isoquercitrin and kaempferitrin), amino acids (methionine, cycstine, tryptophan and lysine) and fatty acids (oleic oils and tocopherols) (Vongsak *et al*., 2013). The presence of diverse bioactive compounds in this highly valued plant may contribute to antihypertensive, antispasmodic, hepatoprotective, antitumour and antimicrobial pharmacological activities observed (Stohs & Hartman, 2015). In view of the interesting phytochemical compounds that it possesses and the abundant availability of this plant, the Ministry of Agriculture (MOA) Malaysia had listed *M. oleifera* as one of the eighteen valuable herbs that need to be studied further and commercialised (Ministry of Agriculture (MOA), 2018). Improvements in the health care system and increased awareness about the importance of conventional medicines have seen many Malaysians seek treatments using conventional drugs. However, a study by Ithnain *et al*. (2020) showed diabetic patients at government clinics in Negeri Sembilan are still taking herbal medicines together with conventional drugs. Similar findings also were observed by Basri *et al*. (2022) for patients with metabolic syndrome at primary care clinics in Selangor. Herbal medicines are a mixture of more than one active ingredient and therefore the likelihood of herb-drug interactions is high (Awortwe *et al*., 2018).

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Phytochemicals in herbal medicines may be substrates for enzymes or transporters that act on drugs, potentially inhibiting metabolism or transport of drugs (Li *et al*., 2014). Moreover, the bioactive constituents of herbal medicines might interact with receptors inducing production of enzymes or transporters (Koe *et al*., 2014) which can alter the absorption, distribution, metabolism and elimination of drugs resulting in toxicity or reduced efficacy (Izzo *et al*., 2016). Studies on possible interactions of Malaysian herbs with conventional antihypertensive agents need to be undertaken to ensure the safety of patients who are taking conventional drugs concurrently with herbal supplements.

Calcium channel blockers (CCBs) are the most prescribed antihypertensive agent in Malaysia (Ng *et al*., 2020). Amlodipine, a CCB, acts by inhibiting the voltage-dependent L-type calcium ion (Ca^{2+}) channels which in return blocks the influx of Ca^{2+}. The low level of intracellular calcium reduces the contractility of vascular smooth muscles, promotes greater relaxation of smooth muscles and improves vasodilation resulting in the drop of the blood pressure (Ferrari *et al*., 2021). A pharmacokinetic study on concomitant oral administration of amlodipine and *Ginkgo biloba* L. (ginkgo) leaf extract in rats showed the inhibition of amlodipine metabolism by the extract. This might due to the modulation of the CYP3A4 metabolic enzyme by *G. biloba* (Wang *et al*., 2016). The CYP3A4 enzyme was also shown to be modulated by *Curcuma* L. (turmeric) when administered together with amlodipine (Jiang *et al*., 2020). Synergistic effect of blood pressure reduction was observed when amlodipine was administered concomitantly with *Zingiber officinale* Roscoe (ginger), *Hibiscus sabdariffa* L. (roselle) (Alam *et al*., 2021) and *Lepidium sativum* L. (garden cress) (Alam *et al*., 2022) respectively.

In this study, the effect of *M. oleifera* leaf extract and its compounds given alone or in combination with amlodipine on HAoSMCs is reported. This study was designed to investigate the effect of *M. oleifera* leaves extract and its compounds on the influx of the extracellular Ca^{2+} into the cytosol and the release of intracellular Ca^{2+} from the endoplasmic reticulum into the cytosol induced by caffeine and K^+ depolarisation. Information gathered from this study is important in understanding the mechanism of blood pressure reduction by *M. oleifera* leaves extract and its compounds and the effect when the extract and its compounds are given together with amlodipine.

MATERIALS AND METHODS

Solvents and chemicals

Foetal bovine serum (FBS), gentamicin sulphate/amphotericin (GA-1000), human aortic smooth muscle cells (HAoSMCs), human epidermal growth factor (hEGF), human fibroblast growth factor-beta (hFGF-ß), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), smooth muscle cell growth medium (SmGM) and trypsin/ ethylenediaminetetraacetic acid (EDTA) were purchased from Lonza, USA. Amlodipine was purchased from Carbosynth, USA. Caffeine, chlorogenic acid, gallic acid, kaempferol, 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) powder, quercetin and vicenin-2 were purchased from Sigma Aldrich, Germany. Dimethyl sulphoxide (DMSO) was purchased from Merck, Germany. Quin 2-AM was purchased from Cayman, USA. $CaC1_2$, glucose, KCl, $MgC1_2$ and NaCl were purchased from Merck Milipore, USA, Triton X-100 was purchased from Nacalai Tesque Inc., Japan. Ethylene glycol tetraacetic acid (EGTA) was purchased from Carbosynth, USA.

Preparation of plant extracts

The fresh leaves of *M. oleifera* were collected from a farm in Kuala Terengganu, Terengganu in October 2020. The plant was identified and authenticated by the Emeritus Professor Dato' Dr Abdul Latif Mohamad of the Faculty of Science and Technology, University Kebangsaan Malaysia (UKM), and a voucher specimen (HF-133) was deposited in the UKM Herbarium. The leaves were cut into small pieces, dried, ground and stored at room temperature (29°C). The ground *M. oleifera* leaf was extracted by boiling the leaf (0.1kg) in distilled water (1.7L) for 6h. The extract was filtered and stored at -80°C for 3 days prior to the freeze-drying and the powdered extract obtained was stored at 4°C until further use (Rathi *et al*., 2006). Before storage, the extract was weighed and the yield was calculated. Other test samples in this study; chlorogenic acid, gallic acid, kaempferol, quercetin and vicenin were chosen based on previous work by the research group (Chin *et al*., 2018; Kumolosasi *et al*., 2022).

Cell culture

HAoSMCs were cultured on T25 flasks in SmGM supplemented with 10% FBS (25 mL), GA-1000 (0.5 mL), hEGF (0.5 mL), hFGF-ß (1 mL) and insulin (0.5 mL). Cells were maintained at 37ºC in a humidified atmosphere with 95% air and of 5% CO_2. The growth medium was replaced once in two days

until cells reached 70 – 80% cell confluency. For experiments, cells were harvested by incubation with 0.25% Trypsin-EDTA and sub-cultured (3500 cells/cm^2) on T25 flasks (Lonza, 2011).

Cell viability assay

MTT assay was used as an indicator of the cells sensitivity to test samples and the positive control. Cells were grown in 96-well plates at a density of 1×10^4 cells per well. After 24 h, the cells were washed with fresh medium and treated with test samples (chlorogenic acid; 3.55, 17.75, 35.50 and 70.86 µg/mL, gallic acid; 1.7 and 8.5 µg/mL, kaempferol; 0.64 and 3.20 µg/mL, *M. oleifera* leaves extract; 20, 40, 80 and 160 µg/mL, quercetin; 3, 15, 30 and 60 µg/mL and vicenin-2; 6 and 30 µg/mL) and positive control (amlodipine; 10, 20, 40, 80 and 160 µg/mL). The cells were incubated for 24 h, washed and 10 µL of MTT solution (5 mg/mL) was added and the mixture was incubated further for 4 h. Next, DMSO (100 µL) was added to dissolve the formazan salt formed. The amount of formazan salt was determined by measuring the optical density (OD) at 570 nm using a microplate reader (Thermo Fisher Scientific, USA) (Hussain Ali *et al*., 2022).

Calcium influx/release blocking assays

On day 6-7, just before confluency was reached, the cells were seeded into 96-well plates at a density of 1×10^4 cells per well and incubated for 48 h in SmGM. Next, the media was aspirated and test samples [chlorogenic acid (3.5 µg/mL), gallic acid (0.8 µg/mL), kaempferol (1.4 µg/mL), *M. oleifera* leaves extract (80 µg/mL), quercetin (1.8 µg/mL) and vicenin-2 (2.9 µg/mL)], amlodipine (5 µg/mL). These solutions were prepared by dissolving those compounds in DMSO and subsequently diluted in serum-free media, ensuring that the highest treatment concentration of DMSO did not exceed 0.1%. A mixture of amlodipine and test samples were added and the cells were incubated further for 30 min. Next, the supernatant was discarded and the cells were washed with fresh media and were incubated with quin 2-AM (50 µM) for 1 h. The cells were rinsed with media (200 µL x 3 times) to remove any residual dye in the extracellular space and were incubated further with normal physiological saline solution (NPSS) for 30 min. A millimolar of NPSS contained $CaC1_2$ (1 mM), glucose (5.5 mM), HEPES (10 mM), KCl (5 mM), $MgC1_2$ (1 mM) and NaCl (135 mM). The NPSS was then discarded and 0.1% Triton X-100 (100 µL) was added (Kanaide *et al.*, 1988). The fluorescence intensity was observed using a microplate reader at 495 nm. This was the procedure for Experiment A where the effect of test samples on cytosolic Ca^{+2} transients in the presence of 1 mM extracellular Ca^{+2} was investigated. To investigate the effect of test samples on cytosolic Ca^{+2} transients in the absence of extracellular Ca^{+2} (Experiment B), ethylene glycol tetraacetic acid (EGTA) (2 mM) was added to NPSS to prepare a millimolar of Ca^{2+} free PSS. For Experiments C and D, caffeine (10 mM) and K^+ (100 mM) were added respectively after the cells were incubated with quin 2-AM. A millimolar of high-K^+ of NPSS was made by replacing NaCl with KCl. The cells were incubated with caffeine for 2 min and K^+ for 4 min (Figure 1).

The most appropriate time to add caffeine and K^+ to the cells was determined. Cells were cultured as described. On day 6 and 7, just before confluency was reached, cells were seeded into 96-well plates at a density of 1×10^4 cells per well and incubated for 48 h. The media was aspirated and quin 2-AM (50 µM) was added and the cells were incubated further in the dark for 1 h. Next, caffeine (10 mM) or K^+ (100 mM) were added at different time points (180, 120, 60, 30, 15 and 0 sec for caffeine) and (10, 8, 6, 4, 2 and 0 min for K^+). Subsequently the plate was put on ice, the supernatant was discarded and 0.1% Triton X-100 (100 µL) was added. The fluorescence intensity was observed using a microplate reader.

Statistical analysis

Data are presented as mean + SEM and values obtained were statistically analysed using GraphPad Prism 7.0 program. The statistical comparisons were made using paired sample t-test, one-way ANOVA and Bonferroni post hoc analyses. Significance was set at $p < 0.05$. The assays were carried out in triplicate.

RESULTS AND DISCUSSION

Cell viability assay

The cell viability assay was carried out to evaluate the effect of test samples (*M. oleifera* leaf extract, chlorogenic acid, gallic acid, kaempferol, quercetin and vicenin-2) and positive control (amlodipine) on cell viability of HAoSMCs. At 80 µg/mL of *M. oleifera* leaves extract, 3.5 µg/mL of chlorogenic acid, 0.8 µg/mL of gallic acid, 1.4 µg/mL of kaempferol, 1.8 µg/mL of quercetin, 2.9 µg/mL of vicenin-2 and 5 µg/mL of amlodipine, HAoSMCs were viable (>85%) after 24 h of incubation. These concentrations were used to treat the cells for the calcium influx/release blocking assays in Experiment A-D (Figure 1).

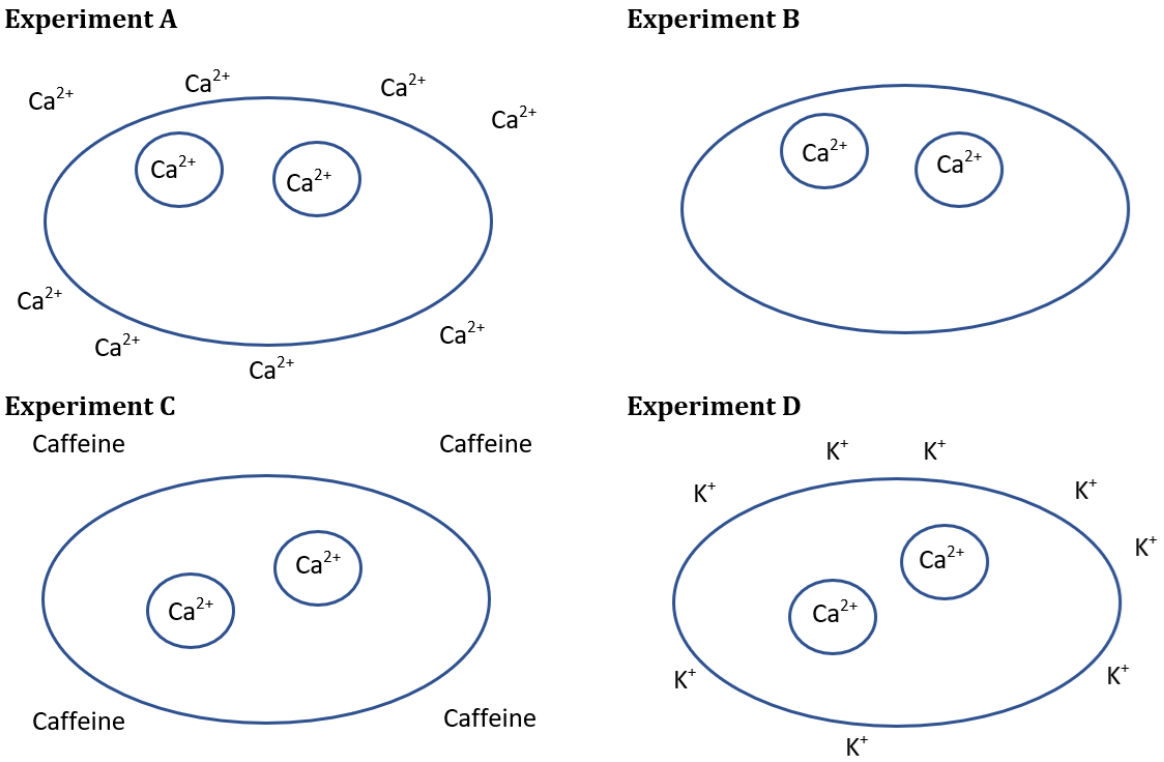

Figure 1. (Experiment A); the effect of *M. oleifera* leaves extract and its compounds on cytosolic Ca^{+2} transients in the presence of extracellular Ca^{+2}. (Experiment B); the effects of *M. oleifera* leaves extract and its compounds on cytosolic Ca^{+2} in the absence of extracellular Ca^{+2}. (Experiment C); the effect of *M. oleifera* leaves extract and its compounds on the release of Ca^{2+} from the sarcoplasmic reticulum induced by caffeine. (Experiment D); the effect of *M. oleifera* leaves extract and its compounds on the release of Ca^{2+} from the sarcoplasmic reticulum induced by K^{+} depolarisation.

Calcium influx/release blocking assays

Ca^{2+} ions play a crucial role in the contraction of all muscle cell types and is a key factor in the excitation-contraction coupling of smooth muscle cells. Vascular smooth muscle cells (VSMCs) contraction appear to be regulated by the changes in cytoplasmic Ca^{2+} concentrations (Imtiaz *et al*., 2006). Influx of extracellular Ca^{2+} through the receptor-operated Ca^{2+} channels (ROCC) and the voltage-dependent Ca^{2+} channels (VDCC) and the release of Ca^{2+} from the sarcoplasmic reticulum by the activation of inositol 1,4,5 triphosphate (IP3) and the ryanodine receptors (RyRs) result in an increase of the intracellular Ca^{2+} leading to contraction (Niazmand *et al*., 2014; Wellman & Nelson, 2003). A key component in achieving vasorelaxation is by lowering the level of intracellular Ca^{2+} (Endo *et al*., 1970).

The effect of *M. oleifera* leaf extract and its compounds on the influx of extracellular Ca^{2+} into the cytoplasm was investigated in Experiment A. Fluorescence intensity of quin2-Ca^{2+} complex decreased significantly in the presence of amlodipine (3.50 ± 0.41), chlorogenic acid (3.19 ± 0.48), gallic acid (2.42 ± 0.42), kaempferol (2.74 ± 0.30), *M. oleifera* leaf extract (3.03 ± 0.57), quercetin (2.86 ± 0.30), vicenin-2 (2.67 ± 0.25) and the combination of amlodipine with test compounds [chlorogenic acid (3.23 ± 0.15), gallic acid (2.37 ± 0.35), kaempferol (3.55 ± 0.29), *M. oleifera* leaf extract (3.22 ± 0.31), quercetin (3.20 ± 0.60), vicenin-2 (3.41 ± 0.37) (Figure 2). However, there was no significant difference observed in the reduction of fluorescence intensity for combination of *M. oleifera* leaf extract and its compounds with amlodipine. This suggest that $Ca^{2\pm}$ influx was blocked by *M. oleifera* leaf extract and its compounds and the combination of *M. oleifera* leaf extract and its compounds with amlodipine but the co-administration did not intensify the $Ca^{2\pm}$ inhibition.

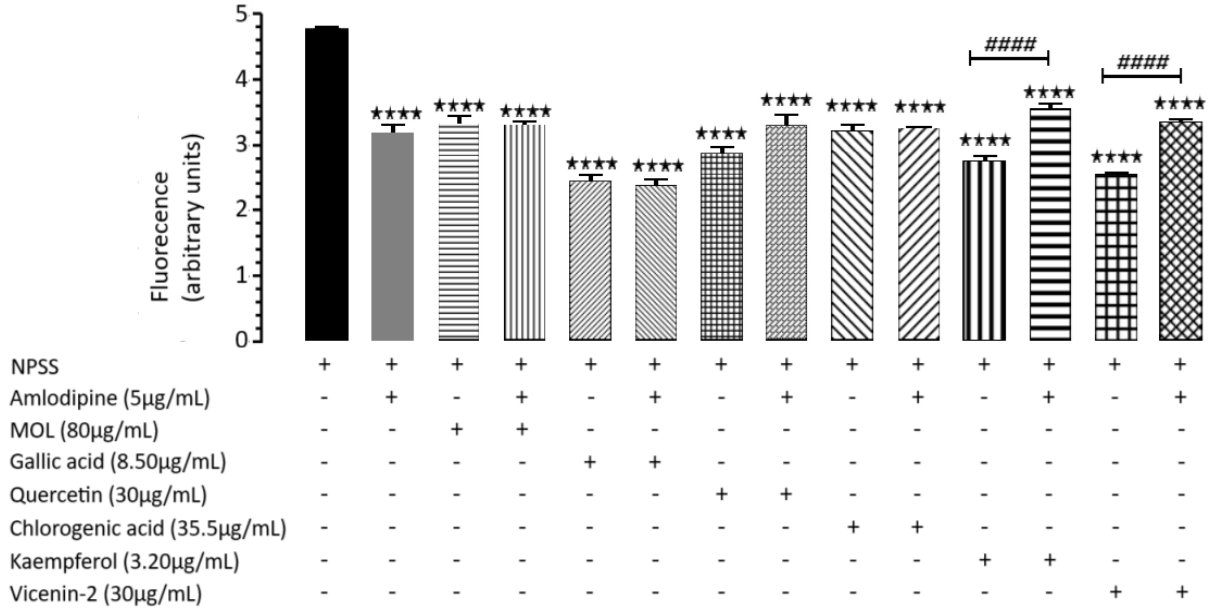

Figure 2. Effect of *M. oleifera* leaves extract and its compounds on cytosolic Ca^{2+} transients in the presence of 1 mM extracellular Ca^{2+}. Each bar shows the triple mean value expressed in mean ± SEM (n = 3), ****p <0.001 and ###p <0.01 compared with negative control.

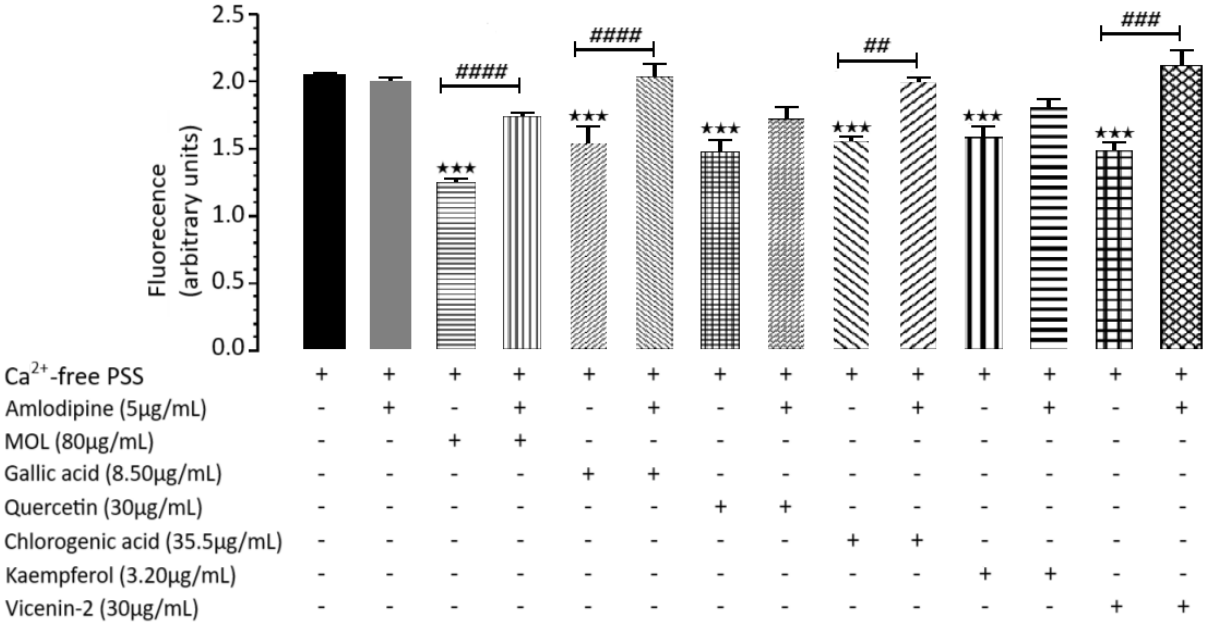

Figure 3. Effect of *M. oleifera* leaves extract and its compounds on cytosolic Ca^{2+} transients in Ca^{2+} free PSS containing 2 mM EGTA. Each bar shows the triple mean value expressed in mean ± SEM (n = 3), *** p <0.001 when compared with negative control, ## p <0.01 and ### p <0.001 when compared with each individual compound.

These data are consistent with previous findings and support the hypothesis that *M. oleifera* leaf extract and its compounds block $Ca^{2\pm}$ channel and therefore relax the vascular smooth muscle (Aekhthammarat *et al*., 2020). Similar blocking effect was demonstrated by *Z. officinale* (ginger) extract and marrubenol, a compound extracted from *Marrubium vulgare* (lotus) (El-Bardai *et al*., 2004; Ghayur & Gilani, 2005). The inhibition of extracellular Ca^{2+} influx by gallic acid (de Oliveira *et al*., 2016; Zhao *et al*., 2012), kaempferol, quercetin (Maaliki *et al*., 2019) and vicenin-2 (Dib *et al*., 2017) is in agreement with previous studies. The absence of additive effect of *M. oleifera* leaf extract and its compounds when used in combination with amlodipine was also seen in the previous work by the research group. It was reported that here was no additive effect of blood pressure lowering activity of *M. oleifera* leaf extract when used in combination with antihypertensive drugs including amlodipine (Kumolosasi *et al*., 2022). This implies the possibility of interaction between *M. oleifera* leaf extract and its compounds with amlodipine.

In Experiment B, the effect of *M. oleifera* leaf extract and its compounds on the release of Ca^{2+} from the sarcoplasmic reticulum was observed. The fluorescence intensity of quin2-Ca^{2+} complex decreased significantly in the presence of chlorogenic acid (1.53 ± 0.23), gallic acid (1.55 ± 0.36), kaempferol (1.52 ± 0.85), *M. oleifera* leaf extract (1.26 ± 0.08), quercetin (1.49 ± 0.25) and vicenin-2 (1.50 ± 0.13) suggesting the intracellular $Ca^{2\pm}$ release is inhibited. However, no significance difference of the fluorescence intensity was observed in the presence of amlodipine, indicating amlodipine did not inhibit the the release of Ca^{2+} from the sarcoplasmic reticulum. Interestingly, significant increase of the fluorescence intensity was observed for these compounds in combination with amlodipine; chlorogenic acid ($p<0.01$), gallic acid ($p<0.001$), *M. oleifera* leaf extract ($p<0.001$) and vicenin-2 ($p<0.001$) when compared with individual compounds (Figure 3) suggesting the inhibitory effect of those compounds was nullified by amlodipine. This antagonistic effect observed might be due to an interaction between amlodipine with these compounds. The results suggest that those compounds exhibit higher potency in inhibiting the release of Ca^{2+} from the sarcoplasmic reticulum when used alone rather than in combination with amlodipine.

The effect of *M. oleifera* leaves extract and its compounds on the release of Ca^{2+} from the sarcoplasmic reticulum induced by caffeine and K^+ depolarisation was studied in Experiment C and D. Prior to the experiment, the optimal time for the release of Ca^{2+} from the sarcoplasmic reticulum induced by caffeine and K^+ depolarisation was determined. The times chosen for caffeine and K^+ were 2 minutes and 4 minutes respectively. For both experiments, the fluorescence intensity of quin2-Ca^{2+} complex decreased significantly in the presence of amlodipine (caffeine-induced; 3.25 ± 0.57, K^+-induced; 3.30 ± 0.63), chlorogenic acid (caffeine-induced; 3.49 ± 0.36, K^+-induced; 3.16 ± 0.33), gallic acid (caffeine-induced; 4.10 ± 0.23, K^+-induced; 2.57 ± 0.23), kaempferol (caffeine-induced; 3.56 ± 0.45, K^+-induced; 2.53 ± 0.37), *M. oleifera* leaf extract (caffeine-induced; 2.98 ± 0.46, K^+-induced; 3.42 ± 0.36), quercetin (caffeine-induced; 2.72 ± 0.33, K^+-induced; 2.42 ± 0.51), vicenin-2 (caffeine-induced; 2.73 ± 0.85, K^+-induced; 2.5 ± 0.77) and the combination of amlodipine with test compounds; chlorogenic acid (caffeine-induced; 3.82 ± 0.47, K^+-induced; 3.43 ± 0.35), gallic acid (caffeine-induced; 4.04 ± 0.36, K^+-induced; 2.76 ± 0.36), kaempferol (caffeine-induced; 4.20 ± 0.47, K^+-induced; 4.04 ± 0.64), *M. oleifera* leaf extract (caffeine-induced; 3.67 ± 0.28, K^+-induced; 3.72 ± 0.41), quercetin (caffeine-induced; 3.20± 0.37, K^+-induced; 3.37 ± 0.31), vicenin-2 (caffeine-induced; 4.14 ± 0.42, K^+-induced; 3.98 ± 0.31) (Figure 4 and 5). Caffeine and K^+ depolarisation induce the releases of Ca^{2+} from the sarcoplasmic reticulum by activating RyRs (Echeverri *et al*., 2010; Kirschstein *et al*., 2009). Caffeine also increases the sensitivity of Ca^{2+}-induced Ca^{2+} release mechanism (CICR) resulting in the rise of cytosolic free Ca^{2+} and thus transient contraction. This reaction is not affected by the extracellular Ca^{2+} and Ca^{2+} channel blockers (Echeverri *et al*., 2010). Results obtained in the Experiment C and D suggest that *M. oleifera* leaf extract and its compounds blocked the release of intracellular Ca^{2+} from the sarcoplasmic reticulum induced by both caffeine and K^+ depolarisation. Interestingly, amlodipine was also seen to inhibit the release of intracellular Ca^{2+} induced by both caffeine and K^+ depolarisation. The inhibitory effect was not seen in Experiment B. It is hypothesised that the action of amlodipine might be due to the feedback loops mechanism to maintain homeostasis (Kholodenko *et al*., 2021).

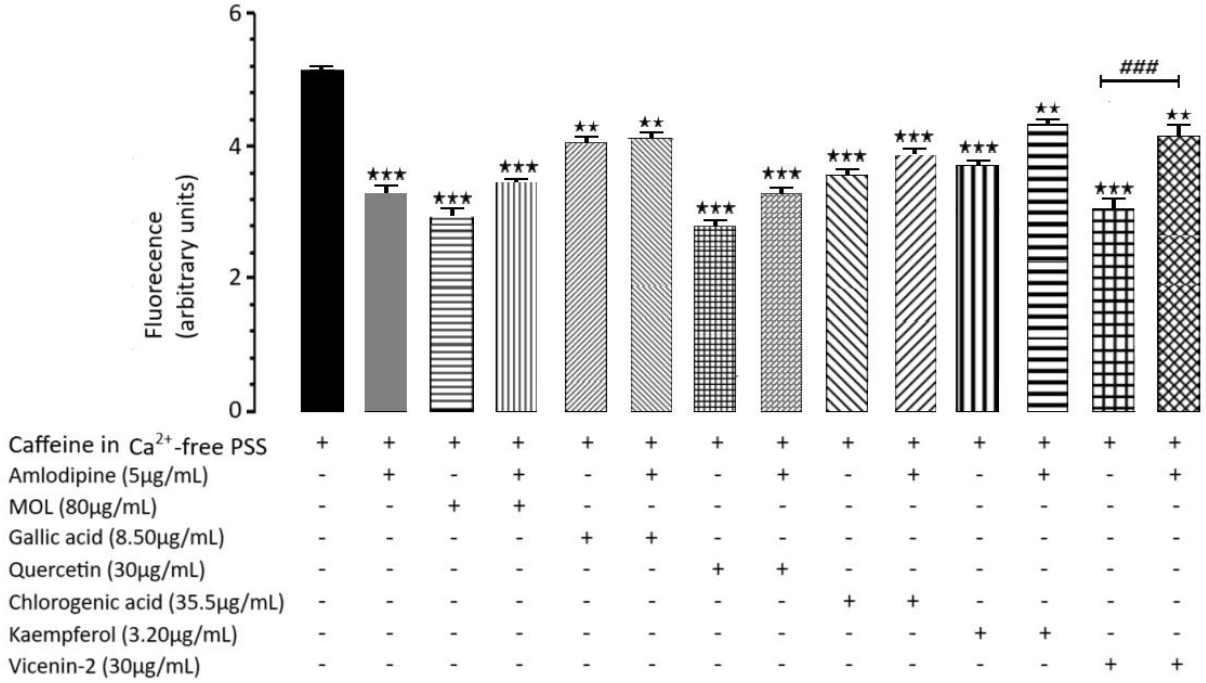

Figure 4. Effect of *M. oleifera* leaves extract *and its compounds* on cytosolic Ca^{2+} transients induced by caffeine. Each bar shows the triple mean value expressed in mean ± SEM (n = 3), ** p <0.01, *** p <0.001 compared with negative control, ### p <0.001 when compared with each individual compound.

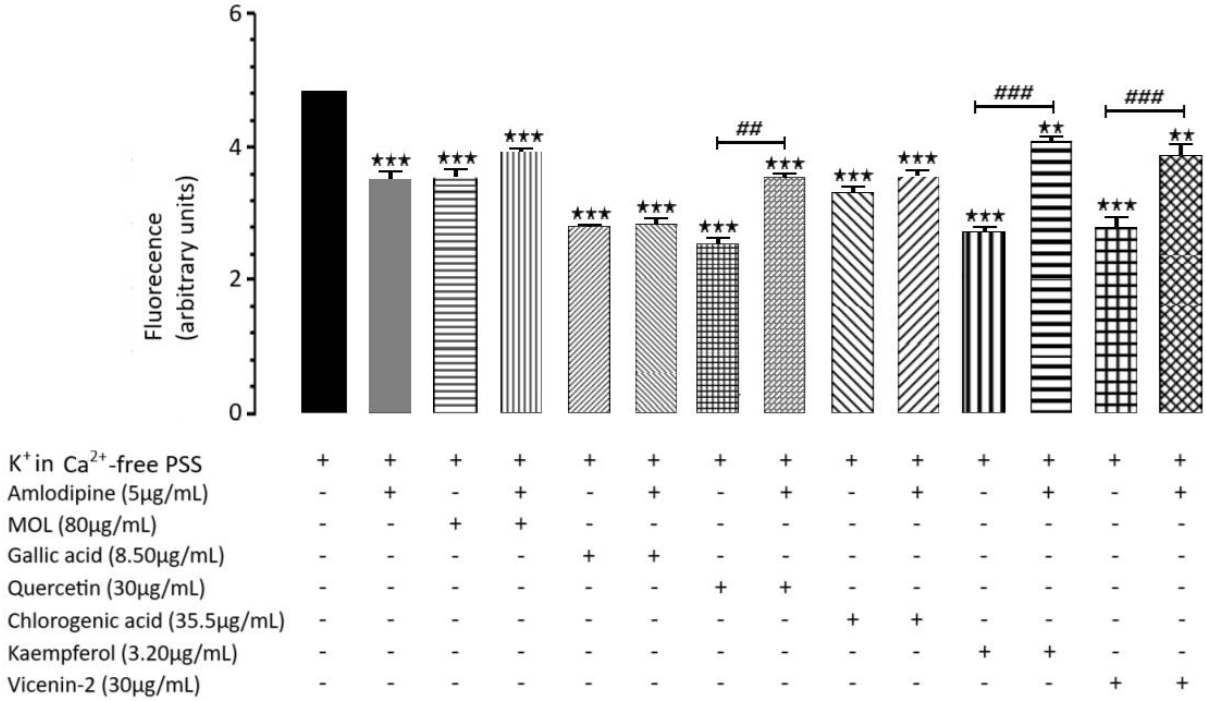

Figure 5. Effect of *M. oleifera* leaves extract and its compounds on cytosolic Ca^{2+} transients induced by K^+ - depolarization. Each bar shows the triple mean value expressed in mean ± sem (n = 3). ** p <0.01, *** p <0.001 compared with negative control, ## p <0.01, ### p <0.001.

Moreover, significant increase of the fluorescence intensity was observed for caffeine-induced; vicenin-2 ($p<0.001$) combined with amlodipine (Figure 4) and K^+-induced; kaempferol ($p<0.001$), quercetin ($p<0.01$) and vicenin-2 ($p<0.001$) combined with amlodipine when compared with the individual compound (Figure 5) suggesting amlodipine antagonised the effect of these compounds in inhibiting calcium release induced by caffeine and K^+ depolarisation.

However, it is conclusive whether blocking of the extracellular Ca^{2+} influx by *M. oleifera* leaf extract and its compounds (Experiment A) was mediated through the ROCC or the VDCC and whether the blocking of Ca^{2+} release from the sarcoplasmic reticulum (Experiment B, C and D) was mediated through the activation of IP3 or RyRs.

CONCLUSION

This study demonstrated that *M. oleifera* leaves extract and its compounds may inhibit intracellular calcium release through several mechanism such as blocking the influx of extracellular Ca^{2+} and blocking the release of intracellular Ca^{2+} from the sarcoplasmic reticulum in cells. When used in combination with amlodipine, the antagonistic effect in intracellular calcium release was observed for some compounds. Further studies are required to understand the ion channels and receptors involved in blocking the intracellular calcium release. Detailed pharmacokinetics study on the effect of *M. oleifera* leaves extract and its compounds when given together with antihypertensive agents will give better understanding on the possible drug-herb interactions which might occur when *M. oleifera* supplements are taken together with antihypertensive agents.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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