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Morinda citrifolia Inhibits Both Cytosolic Ca^{2+}-dependent Phospholipase A_2 and Secretory Ca^{2+}-dependent Phospholipase A_2

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This study investigated the effects of the methanol extracts of *Morinda citrifolia* containing numerous anthraquinone and iridoid on phospholipase A_2 (PLA_2) isozyme. PLA_2 activity was measured using various PLA_2 substrates, including 10-pyrene phosphatidylcholine, 1-palmitoyl-2-[^{14}C]arachidonyl phosphatidylcholine ([^{14}C]AA-PC), and [^{3}H]arachidonic acid (AA). The methanol extracts suppressed melittin-induced [$_{3}$H]AA release in a concentration-dependent manner in RAW 264.7 cells, and inhibited $cPLA_2$/$sPLA_2$-induced hydrolysis of [^{14}C]AA-PC in a concentration- and time-dependent manner. A Dixon plot showed that the inhibition by methanol extracts on $cPLA_2$ and $sPLA_2$ appeared to be competitive with inhibition constants (K_i) of 3.7 μg/ml and 12.6 μg/ml, respectively. These data suggest that methanol extracts of *Morinda citrifolia* inhibits both Ca^{2+}-dependent PLA_2 such as, $cPLA_2$ and $sPLA_2$. Therefore, *Morinda citrifolia* may possess anti-inflammatory activity secondary to Ca^{2+}-dependent PLA_2 inhibition.

Key Words: *Morinda citrifolia*, Phospholipase A_2, Arachidonic acid

INTRODUCTION

Phospholipase A_2 (PLA_2) plays an important role in inflammatory responses through hydrolysis of cell membrane phospholipids. This leads to arachidonic acid and lysophospholipid production [1]. PLA_2 isozymes are classified according to their nucleotide sequences and are classified into three types secretory PLA_2 ($sPLA_2$), cytosolic Ca^{2+}-dependent PLA_2 ($cPLA_2$), and Ca^{2+}-independent PLA_2 ($iPLA_2$) based on cellular activity and functions [2]. $sPLA_2$ has a low molecular mass (~18 kDa) and can be activated at millimolar concentrations of Ca^{2+}. $sPLA_2$ is secreted by inflammatory cells upon their activation and by damaged tissues in inflammatory diseases [3]. $cPLA_2$ is located in the cytosol, has a high molecular mass (~85 kDa), and can be activated at the micromolar concentration of intracellular Ca^{2+} [4]. $iPLA_2$ can be detected in both cytosolic and membrane fractions with a molecular mass ranging from 29 to 85 kDa, and does not require Ca^{2+} for activation [5].

Morinda citrifolia (Rubiaceae) is widely distributed across Polynesia and contains numerous anthraquinone and iridoids, such as asperuloside, asperulosidic acid and deacetylasperulosidic acid [6]. It has been traditionally used as a folk medicine for the treatment of many disease, including arthritis, diabetes, hypertension, heart disease, neoplasia, and atherosclerosis [7]. Although *Morinda citrifolia* has a wide range of biological activities, there is no evidence of inhibitory effects on PLA_2 isozymes. We measured the effects of the methanol extracts of *Morinda citrifolia* composing major biological active components on PLA_2 isozyme activity in the present study.

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METHODS

Materials

PLA_2 from honey bee venom, arachidonyl trifluoromethyl ketone ($AACOCF_3$), bromoenol lactone (BEL) and melittin, was purchased from the Sigma Chemical Co. (St. Louis, Mo, USA). 10-Pyrene phosphatidylcholine (10-pyrene PC) was purchased from Molecular Probes (Leiden, Netherlands), [^{3}H]arachidonic acid ([^{3}H]AA) was obtained from NEN (Boston, USA), 1-palmitoyl-2-[^{14}C]arachidonyl phosphatidylcholine ([^{14}C]AA-PC) was obtained from Perkin Elmer (Boston, USA), and 1-palmitoyl-2-arachidonyl phosphatidylcholine was obtained from Avanti Polar Lipid (Alabaster, USA).

Cell culture

The RAW 264.7 murine macrophage cell line was obtained from the Korean Cell Line Bank (Seoul, Korea) and were cultured in DMEM supplemented with 10% heat-inactivated FBS and antibiotic-antifungal mix (100 IU/ml penicillin G, 100 μg/ml of streptomycin and 0.25 μg/ml of amphotericin B) at 37°C with 5% CO_2.

ABBREVIATIONS: PLA_2, phospholipase A_2; AA, arachidonic acid; [^{14}C]AA-PC, 1-palmitoyl-2-[^{14}C]arachidonyl phosphatidylcholine; $AACOCF_3$, arachidonyl trifluoromethyl ketone; DTT, dithiothreitol; BEL, bromoenol lactone.

Preparation of methanol extracts

Morinda citrifolia fruit powder was purchased from the NP Nutra Co. (Hawaii). Dried *Morinda citrifolia* fruit powder (800 g) was extracted with 70% methanol at 40°C. It has been previously reported that the methanol extracts contained a numerous antharquinone and iridoids, such as asperuloside, asperulosidic acid and deacetylasperulosidic acid [6,8].

Measurement of [^{3}H]AA release

RAW 264.7 cells were harvested with Krebs buffer (137 mM NaCl, 2.7 mM KCl, 0.4 mM Na_2HPO_4, 0.5 mM $MgCl_2$, 10 mM HEPES (pH 7.4), 1.8 mM $CaCl_2$, and 5 mM glucose). Cells were labeled with [^{3}H]AA (0.4 μCi/ml) at 37°C for 2 h and were washed with Krebs buffer containing 0.5 mg/ml bovine serum albumin to trap the liberated [^{3}H]AA. AA release was induced by 0.5 μM melittin either with or without the methanol extracts for 30 min. Both pellets and supernatants were transferred to liquid scintillation vials after centrifugation. Radioactivity was measured with a liquid scintillation counter [9], and the percentage of [^{3}H]AA release was calculated as supernatants/(supernatants+pellets)×100.

sPLA$_2$ assay with 10-pyrene PC

PLA_2 activity was measured with a pyrene-labeled phosphatidylcholine in the presence of serum albumin [10] using a spectrophotometer. PLA_2 purified from honey bee venom was used as $sPLA_2$ [11]. $sPLA_2$ and the methanol extracts were vortex-mixed and incubated for 10 min at room temperature. The above enzyme was incubated with a reaction mixture containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 2 μM 10-pyrene PC, 0.1% bovine serum albumin, and 6 mM $CaCl_2$ for 20 min. The fluorescence was measured using excitation (345 nm) and emission (398 nm) wavelengths with a spectrophotometer (FL600, Microplate Fluorescence Reader, Bio-Tek).

Preparation of RAW 264.7 cell-derived cPLA$_2$

RAW 264.7 cells were washed and sonicated in 10 mM Tris-HCl buffer (pH 7.4) with 100 mM NaCl, 2 mM EGTA, 100 μM leupeptin, 150 μM aprotinin, and 1 mM Na_3VO_4. Lysates were centrifuged at 10,000 g for 30 min at 4°C, and supernatants were stored at −70°C and used to supply $cPLA_2$ [12].

cPLA$_2$ and sPLA$_2$ assay with [^{14}C]AA-PC

PLA_2 activity was assayed by measurement of [^{14}C]AA hydrolyzed from [^{14}C]AA-PC. For the measurement of RAW 264.7 cell-derived $cPLA_2$ activity, enzyme sources and methanol extracts were incubated for 30 min at room temperature in 100 mM Tris-HCl buffer (pH 8.5) containing 10 μM BEL as the $iPLA_2$ inhibitor [13], and 5 mM $CaCl_2$ and 1 mM dithiothreitol (DTT) as the $sPLA_2$ inhibitors [14]. The reaction mixture was incubated with 0.025 μCi [^{14}C] AA-PC as the substrate for a given time. The $cPLA_2$ inhibitor $AACOCF_3$ (10 μM) [15] was used for the measurement of $sPLA_2$ activity instead of DTT.

The reaction mixture was incubated at 37°C for 30 min. The reaction was stopped by the addition of modified Doles reagent (n-heptane/isopropyl alcohol/1 N-H_2SO_4=400/390/10, 560 μl) [16]. After centrifugation, 150 μl of upper phase was transferred to a new tube; n-heptane (800 μl) and silica gel (10 mg) were added to the tube. The mixtures were mixed and centrifuged for 2 min, and supernatants (800 μl) were mixed with scintillation solution (1.0 ml) and counted for radioactivity in a Packard Tri-carb liquid scintillation counter. The specific activity in picomoles per minute per milligram protein (pmol · min^{-1} · mg^{-1}) was obtained by dividing activity by the amount of enzyme protein. Protein was analyzed with a BCA (bicinchoninic acid) protein assay kit to calculate specific activity. The [^{14}C]AA-PC hydrolysis ratio was applied to compose the Dixon plot, and the concentration of the substrate was increased by adding 1-palmitoyl-2-arachidonyl phosphatidylcholine.

Data analysis

The results are represented as mean±standard deviation (S.D.) values and analyzed statistically by analysis of variance (ANOVA). Differences between groups were determined with the Newman-Keul's test. Statistical significance was defined for p values less than 0.05.

RESULTS

Effects of methanol extracts on melittin-induced AA release

The methanol extracts containing numorous anthraquinone and iridoids, such as asperuloside, asperulosidic acid and deacetylasperulosidic acid, displayed more potent inhibitory effects on melittin-induced AA release in [^{3}H]AA-labeled RAW 264.7 cells than other fractions (data not shown). Melittin (0.5 μM) resulted in AA release by 18.3±2.0%, which was significantly decreased by the methanol extracts at 10 and 100 μg/ml to 14.9±1.8% and 12.5±0.4%, respectively (Fig. 1).

Effects of methanol extracts on cPLA$_2$ activity using [^{14}C]AA-PC

We have previously reported that the RAW 264.7 cell-de-

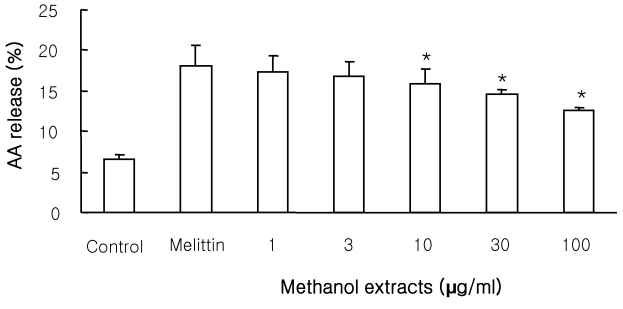

Fig. 1. Effects of the methanol extracts on [^{3}H]arachidonic acid (AA) release in RAW 264.7 cells stimulated by 0.5 μM melittin. Cells were incubated with the methanol extracts at 37°C for 10 min and AA release was induced by 0.5 μM melittin. Results are mean±S.D. values from 4 separate experiments. *$p<0.05$ *vs* melittin.

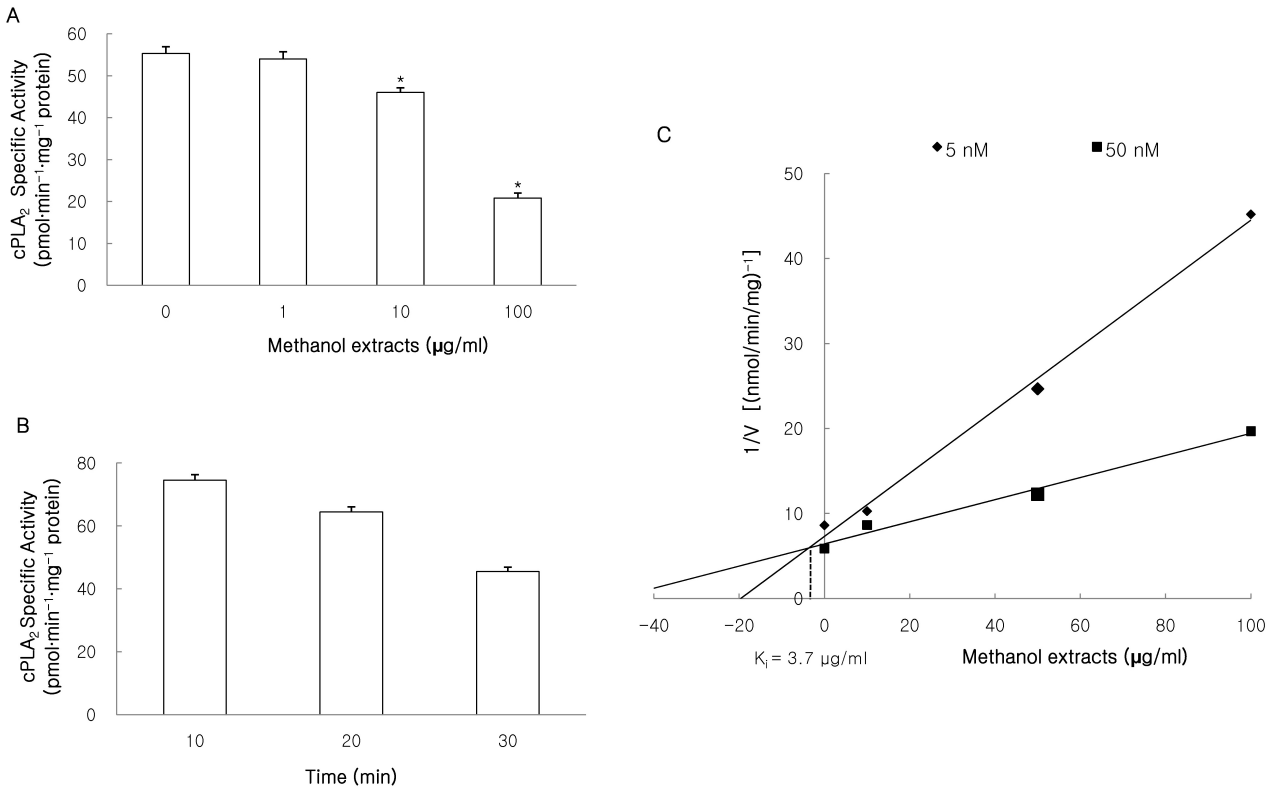

Fig. 2. Effects of methanol extracts on $cPLA_2$ activity. $cPLA_2$ activity was measured using 1-palmitoyl-2-[^{14}C]arachidonyl phosphatidylcholine ([^{14}C]AA-PC) as a substrate by previously methods. The methanol extracts inhibited $cPLA_2$-induced hydrolysis of [^{14}C]AA-PC in a concentration (A)- and time (B)-dependent manner. A Dixon plot showed that $cPLA_2$ inhibition by methanol extracts appeared to be competitive with an inhibition constant (K_i) of 3.7 μg/ml (C). Results are mean±S.D. values from 4 separate experiments. *$p<0.05$ vs control.

rived PLA_2 was significantly inhibited by 10 μM $AACOCF_3$, but was not influenced by 1 mM DTT or 10 μM BEL. Also RAW 264.7 cell-derived PLA_2 did not show any PLA_2 activity in the absence of Ca^{2+} [17]. These data suggest that the RAW 264.7 cell-derived PLA_2 contained mainly $cPLA_2$.

RAW 264.7 cell-derived PLA_2 and PLA_2-specific substrate, [^{14}C]AA-PC were used to investigate the effects of the methanol extracts on $cPLA_2$. The methanol extracts inhibited $cPLA_2$ in a concentration- and time-dependent manner (Fig. 2A and 2B), and inhibited $cPLA_2$ activity by 16.8% and 63.2% at concentrations of 10 and 100 μg/ml, respectively.

Dixon plots were constructed from $cPLA_2$-induced substrate hydrolysis rates ([^{14}C]AA-PC of 5 and 50 nM) at various concentrations of methanol extracts to determine the inhibitory pattern on $cPLA_2$ by methanol extracts. Fig. 2C demonstrated that the apparent K_i value of the methanol extracts on $cPLA_2$ was 3.7 μg/ml, and such an inhibitory pattern suggests that the methanol extracts acted as a competitive inhibitor against $cPLA_2$.

Effects of methanol extracts on $sPLA_2$ using [^{14}C]AA-PC and 10-pyrene PC

$sPLA_2$ obtained from honey bee venom hydrolyzed 10-pyrene PC; the $sPLA_2$-specific substrate [18]. We used purified $sPLA_2$ from honey bee venom to investigate the inhibitory effects of the methanol extracts on $sPLA_2$. The methanol extracts inhibited $sPLA_2$ in a concentration- and time-dependent manner (Fig. 3A and 3B). The methanol extracts inhibited $sPLA_2$ activity by 15.6% and 53.9% at concentrations of 10 and 100 μg/ml, respectively.

Dixon plots were constructed from $sPLA_2$-induced hydrolysis rates of the substrate ([^{14}C]AA-PC of 5 and 50 nM) to determine the $sPLA_2$ inhibitory patterns by the methanol extracts. Fig. 3C demonstrated that the apparent K_i value of the methanol extracts on $sPLA_2$ was 12.6 μg/ml, and such inhibitory patterns suggest that methanol extracts acts as a competitive inhibitor against $sPLA_2$. Additionally, the methanol extracts inhibited bee venom $sPLA_2$-induced 10-pyrene PC hydrolysis in a concentration-dependent manner (Fig. 4).

DISCUSSION

The methanol extracts decreased melittin-induced [^{3}H]AA release in RAW 264.7 cells in a concentration-dependent manner in the present study. Melittin has been used as an endogenous PLA_2 activator by increasing intracellular Ca^{2+} concentrations via receptor-operated calcium channels [19]. Such inhibitory effects of the methanol extracts may be associated with PLA_2 inhibition. However, AA release is an indirect measure of PLA_2 activity since other enzymes including arachidonyl-CoA synthetase, CoA-dependent acyltransferase, and CoA-independent transacylase may re-

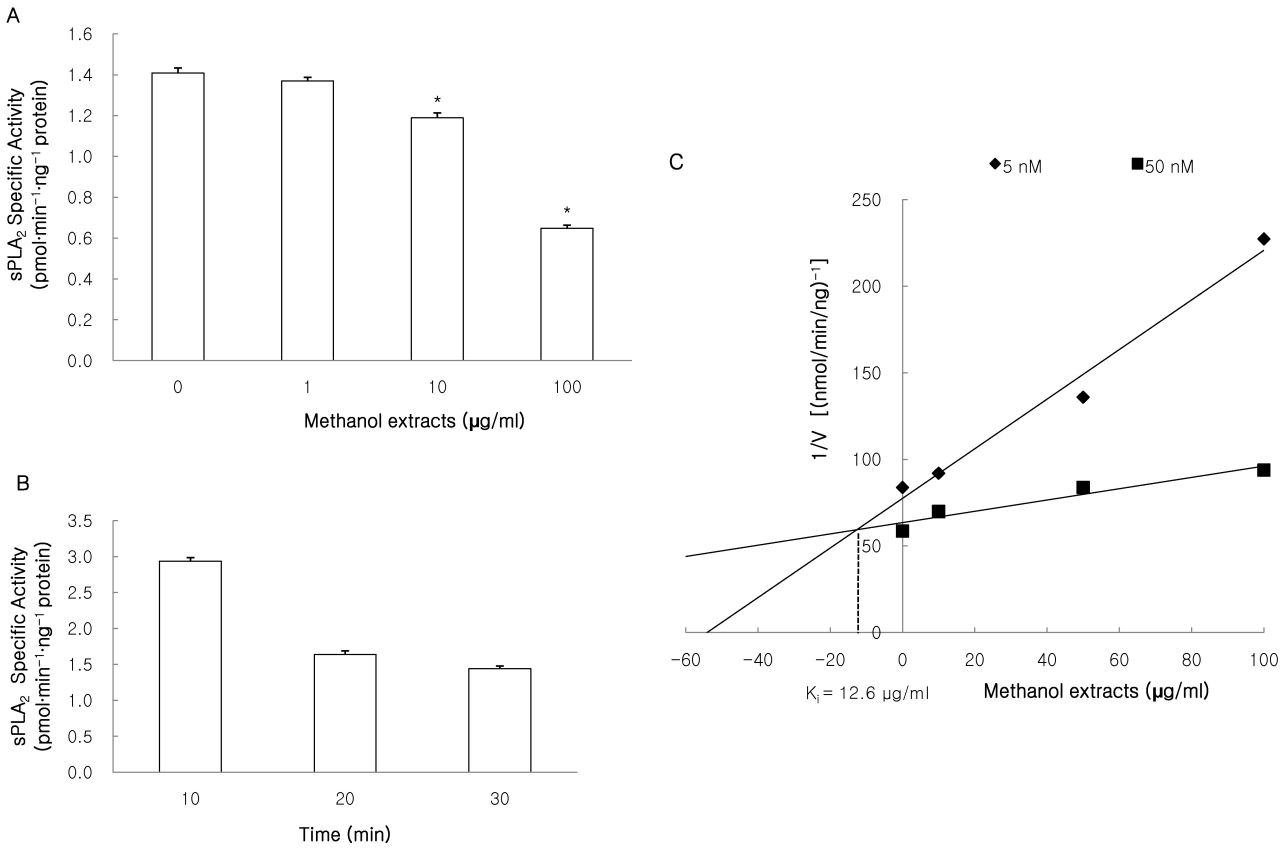

Fig. 3 Effects of methanol extracts on $sPLA_2$ activity. $sPLA_2$ activity was measured using 1-palmitoyl-2-[^{14}C]arachidonyl phosphatidylcholine ([^{14}C]AA-PC) as a substrate by previously described methods. The methanol extracts inhibited $sPLA_2$-induced hydrolysis of [^{14}C]AA-PC in a concentration (A)- and time (B)-dependent manner. A Dixon plot showed that the inhibition of $sPLA_2$ by methanol extracts appeared to be competitive with an inhibition constant (K_i) of 12.6 μg/ml (C). Results are mean±S.D. values from 4 separate experiments. *$p<0.05$ *vs* control.

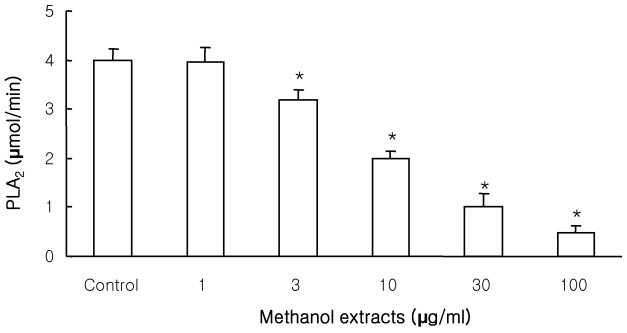

Fig. 4. Effects of methanol extracts on bee venom $sPLA_2$ activity with 10-pyrene phosphatidylcholine (10-Pyrene PC). 10-Pyrene PC hydrolyzed by purified $sPLA_2$ was inhibited by the methanol extracts in a concentration-dependent manner. Results are mean± S.D. values from 4 separate experiments. *$p<0.05$ *vs* control.

sult in free AA production [20]. We measured direct effects of methanol extracts on PLA_2 isozymes using [^{14}C]AA-PC, a specific PLA_2 substrate to address this issue.

RAW 264.7 cellular lysates were used as the source of $cPLA_2$ since we did not prepare each PLA_2 isozyme. RAW 264.7 cell-derived PLA_2 was completely inhibited by $AACOCF_3$ but not by DTT or BEL. RAW cell-derived PLA_2 did not result in any PLA_2 activity using the $sPLA_2$-specific substrate, 10-pyrene PC. Additionally, RAW 264.7 cell-derived PLA_2 showed only a low level of activity in the absence of Ca^{2+} [17]. These data suggested that RAW 264.7 cell-derived PLA_2 primarily contained $cPLA_2$ rather than $sPLA_2$ and $iPLA_2$.

We determined the inhibitory pattern of the methanol extracts on $cPLA_2$ with Dixon plots. The methanol extracts inhibited $cPLA_2$-induced hydrolysis of [^{14}C]AA-PC in a concentration- and time-dependent manner. Dixon plot was constructed to demonstrate that inhibition by the methanol extracts appeared to be competitive with an inhibition constant (K_i) of 3.7 μg/ml. It has been reported that $cPLA_2$ is inhibited by a trifluoromethyl ketone analog of AA ($AACOCF_3$), which presumably binds directly to the active site of $cPLA_2$ [15,21,22]; $AACOCF_3$ (10 μM) inhibited $cPLA_2$ by 95.4%, and the methanol extracts (100 μg/ml) inhibited it by 63.2%. Bee venom $sPLA_2$ was used to investigate the effects of the methanol extracts on $sPLA_2$. The methanol extracts inhibited $sPLA_2$-induced hydrolysis of [^{14}C]AA-PC in a concentration- and time-dependent manner. Dixon plot demonstrated that the inhibition by methanol extracts appeared to be competitive, with an inhibition constant (K_i)

of 12.6 μg/ml. These data suggest that methanol extracts of the *Morinda citrifolia* fruit inhibits both Ca^{2+}-dependent PLA_2 such as $cPLA_2$ and $sPLA_2$. *Morinda citrifolia* anti-inflammatory activity was observed by a locally acute inflammatory response such as, bradykinin-induced rat paw edema [23] and a selective inhibition effect on cyclooxygenase [24]. These data support that the potential anti-inflammatory activity of *Morinda citrifolia* may be secondary to the inhibition of Ca^{2+}-dependent PLA_2.

In conclusion, the methanol extracts of the *Morinda citrifolia* fruit containing numerous anthraquinone and iridoids, such as asperuloside, asperulosidic acid and deacetylasperulosidic acid [6] decreased melittin-induced [^{3}H]AA release in RAW 264.7 cells in a concentration-dependent manner. The methanol extracts inhibited $cPLA_2$/$sPLA_2$-induced hydrolysis of [^{14}C]AA-PC in both a concentration- and time-dependent manner. A Dixon plot demonstrated that $cPLA_2$ and $sPLA_2$ inhibition by the methanol extracts appeared to be competitive with an inhibition constant (K_i) of 3.7 μg/ml and 12.6 μg/ml, respectively. These data suggest that methanol extracts of the *Morinda citrifolia* fruit inhibits both Ca^{2+}-dependent PLA_2, such as $cPLA_2$ and $sPLA_2$. These data supported that the anti-inflammatory activity of *Morinda citrifolia* can be secondary to the inhibition of Ca^{2+}-dependent PLA_2.

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