

## *Morinda citrifolia* Inhibits Both Cytosolic Ca<sup>2+</sup>-dependent Phospholipase A<sub>2</sub> and Secretory Ca<sup>2+</sup>-dependent Phospholipase A<sub>2</sub>

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

**Key Words:** *Morinda citrifolia*, Phospholipase A<sub>2</sub>, Arachidonic acid

### INTRODUCTION

Phospholipase A<sub>2</sub> (PLA<sub>2</sub>) plays an important role in inflammatory responses through hydrolysis of cell membrane phospholipids. This leads to arachidonic acid and lysophospholipid production [1]. PLA<sub>2</sub> isozymes are classified according to their nucleotide sequences and are classified into three types secretory PLA<sub>2</sub> (sPLA<sub>2</sub>), cytosolic Ca<sup>2+</sup>-dependent PLA<sub>2</sub> (cPLA<sub>2</sub>), and Ca<sup>2+</sup>-independent PLA<sub>2</sub> (iPLA<sub>2</sub>) based on cellular activity and functions [2]. sPLA<sub>2</sub> has a low molecular mass (~18 kDa) and can be activated at millimolar concentrations of Ca<sup>2+</sup>. sPLA<sub>2</sub> is secreted by inflammatory cells upon their activation and by damaged tissues in inflammatory diseases [3]. cPLA<sub>2</sub> is located in the cytosol, has a high molecular mass (~85 kDa), and can be activated at the micromolar concentration of intracellular Ca<sup>2+</sup> [4]. iPLA<sub>2</sub> can be detected in both cytosolic and membrane fractions with a molecular mass ranging from 29 to 85 kDa, and does not require Ca<sup>2+</sup> 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<sub>2</sub> isozymes. We measured the effects of the methanol extracts of *Morinda citrifolia* composing major biological active components on PLA<sub>2</sub> isozyme activity in the present study.

### METHODS

#### Materials

PLA<sub>2</sub> from honey bee venom, arachidonyl trifluoromethyl ketone (AACOCF<sub>3</sub>), 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). [<sup>3</sup>H]arachidonic acid ([<sup>3</sup>H]AA) was obtained from NEN (Boston, USA). 1-palmitoyl-2-[<sup>14</sup>C]arachidonyl phosphatidylcholine ([<sup>14</sup>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<sub>2</sub>.

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**ABBREVIATIONS:** PLA<sub>2</sub>, phospholipase A<sub>2</sub>; AA, arachidonic acid; [<sup>14</sup>C]AA-PC, 1-palmitoyl-2-[<sup>14</sup>C]arachidonyl phosphatidylcholine; AACOCF<sub>3</sub>, 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 anthraquinone and iridoids, such as asperuloside, asperulosidic acid and deacetylasperulosidic acid [6,8].

### Measurement of [<sup>3</sup>H]AA release

RAW 264.7 cells were harvested with Krebs buffer (137 mM NaCl, 2.7 mM KCl, 0.4 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.5 mM MgCl<sub>2</sub>, 10 mM HEPES (pH 7.4), 1.8 mM CaCl<sub>2</sub>, and 5 mM glucose). Cells were labeled with [<sup>3</sup>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 [<sup>3</sup>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 [<sup>3</sup>H]AA release was calculated as supernatants/(supernatants + pellets) × 100.

### sPLA<sub>2</sub> assay with 10-pyrene PC

PLA<sub>2</sub> activity was measured with a pyrene-labeled phosphatidylcholine in the presence of serum albumin [10] using a spectrophotometer. PLA<sub>2</sub> purified from honey bee venom was used as sPLA<sub>2</sub> [11]. sPLA<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub>

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<sub>3</sub>VO<sub>4</sub>. 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<sub>2</sub> [12].

### cPLA<sub>2</sub> and sPLA<sub>2</sub> assay with [<sup>14</sup>C]AA-PC

PLA<sub>2</sub> activity was assayed by measurement of [<sup>14</sup>C]AA hydrolyzed from [<sup>14</sup>C]AA-PC. For the measurement of RAW 264.7 cell-derived cPLA<sub>2</sub> 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<sub>2</sub> inhibitor [13], and 5 mM CaCl<sub>2</sub> and 1 mM dithiothreitol (DTT) as the sPLA<sub>2</sub> inhibitors [14]. The reaction mixture was incubated with 0.025 μCi [<sup>14</sup>C] AA-PC as the substrate for a given time. The cPLA<sub>2</sub> inhibitor AACOCF<sub>3</sub> (10 μM) [15] was used for the measurement of sPLA<sub>2</sub> 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<sub>2</sub>SO<sub>4</sub>=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<sup>-1</sup> · mg<sup>-1</sup>) 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 [<sup>14</sup>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.

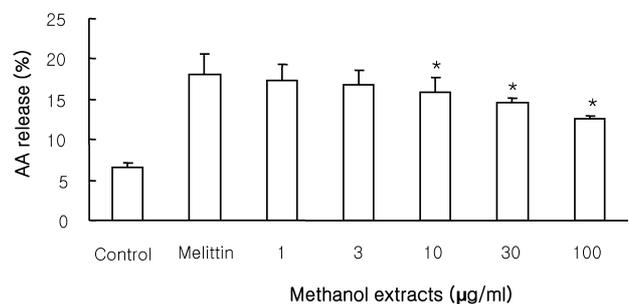
## RESULTS

### Effects of methanol extracts on melittin-induced AA release

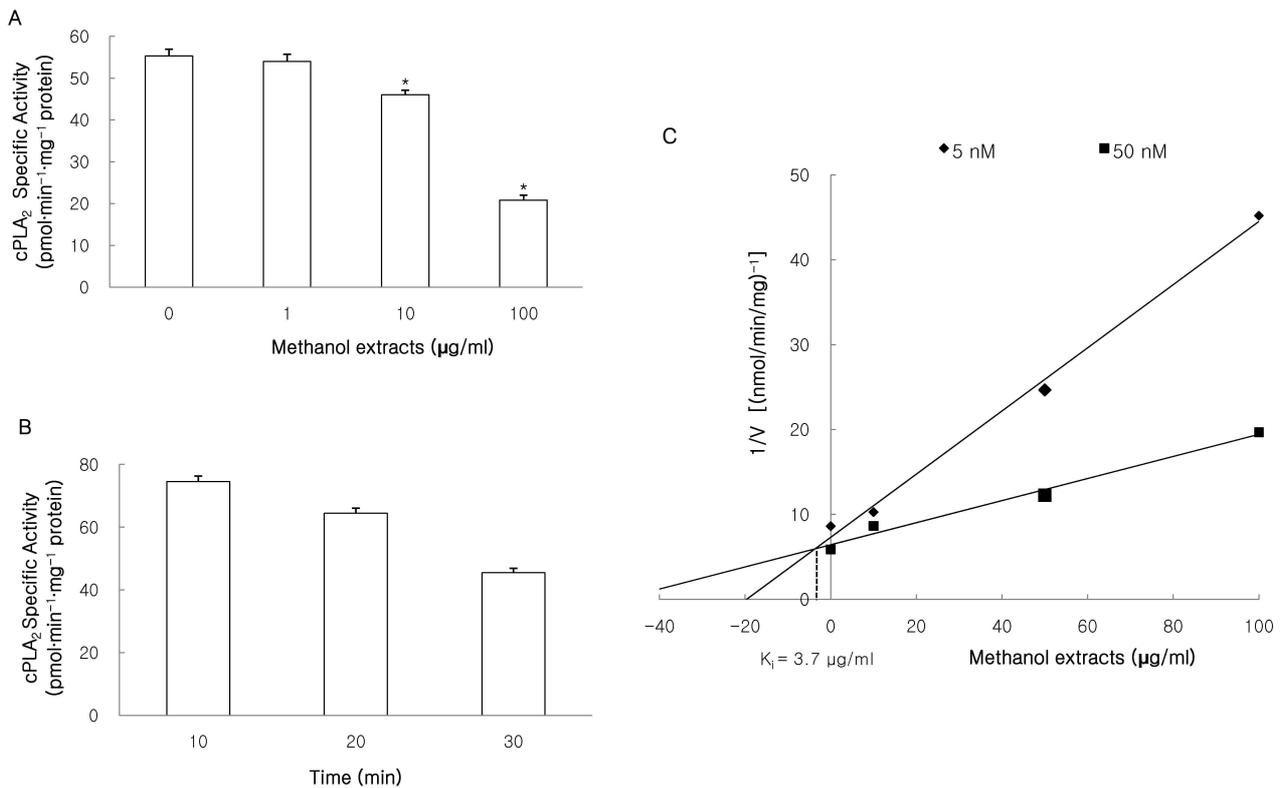
The methanol extracts containing numerous anthraquinone and iridoids, such as asperuloside, asperulosidic acid and deacetylasperulosidic acid, displayed more potent inhibitory effects on melittin-induced AA release in [<sup>3</sup>H]AA-labeled RAW 264.7 cells than other fractions (data not shown). Melittin (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<sub>2</sub> activity using [<sup>14</sup>C]AA-PC

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



**Fig. 1.** Effects of the methanol extracts on [<sup>3</sup>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<sub>2</sub> activity. cPLA<sub>2</sub> activity was measured using 1-palmitoyl-2-[<sup>14</sup>C]arachidonyl phosphatidylcholine ([<sup>14</sup>C]AA-PC) as a substrate by previously methods. The methanol extracts inhibited cPLA<sub>2</sub>-induced hydrolysis of [<sup>14</sup>C]AA-PC in a concentration (A)- and time (B)-dependent manner. A Dixon plot showed that cPLA<sub>2</sub> inhibition by methanol extracts appeared to be competitive with an inhibition constant ( $K_i$ ) of 3.7  $\mu\text{g/ml}$  (C). Results are mean  $\pm$  S.D. values from 4 separate experiments. \* $p < 0.05$  vs control.

rived PLA<sub>2</sub> was significantly inhibited by 10  $\mu\text{M}$  AACOCF<sub>3</sub>, but was not influenced by 1 mM DTT or 10  $\mu\text{M}$  BEL. Also RAW 264.7 cell-derived PLA<sub>2</sub> did not show any PLA<sub>2</sub> activity in the absence of  $\text{Ca}^{2+}$  [17]. These data suggest that the RAW 264.7 cell-derived PLA<sub>2</sub> contained mainly cPLA<sub>2</sub>.

RAW 264.7 cell-derived PLA<sub>2</sub> and PLA<sub>2</sub>-specific substrate, [<sup>14</sup>C]AA-PC were used to investigate the effects of the methanol extracts on cPLA<sub>2</sub>. The methanol extracts inhibited cPLA<sub>2</sub> in a concentration- and time-dependent manner (Fig. 2A and 2B), and inhibited cPLA<sub>2</sub> activity by 16.8% and 63.2% at concentrations of 10 and 100  $\mu\text{g/ml}$ , respectively.

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

#### Effects of methanol extracts on sPLA<sub>2</sub> using [<sup>14</sup>C]AA-PC and 10-pyrene PC

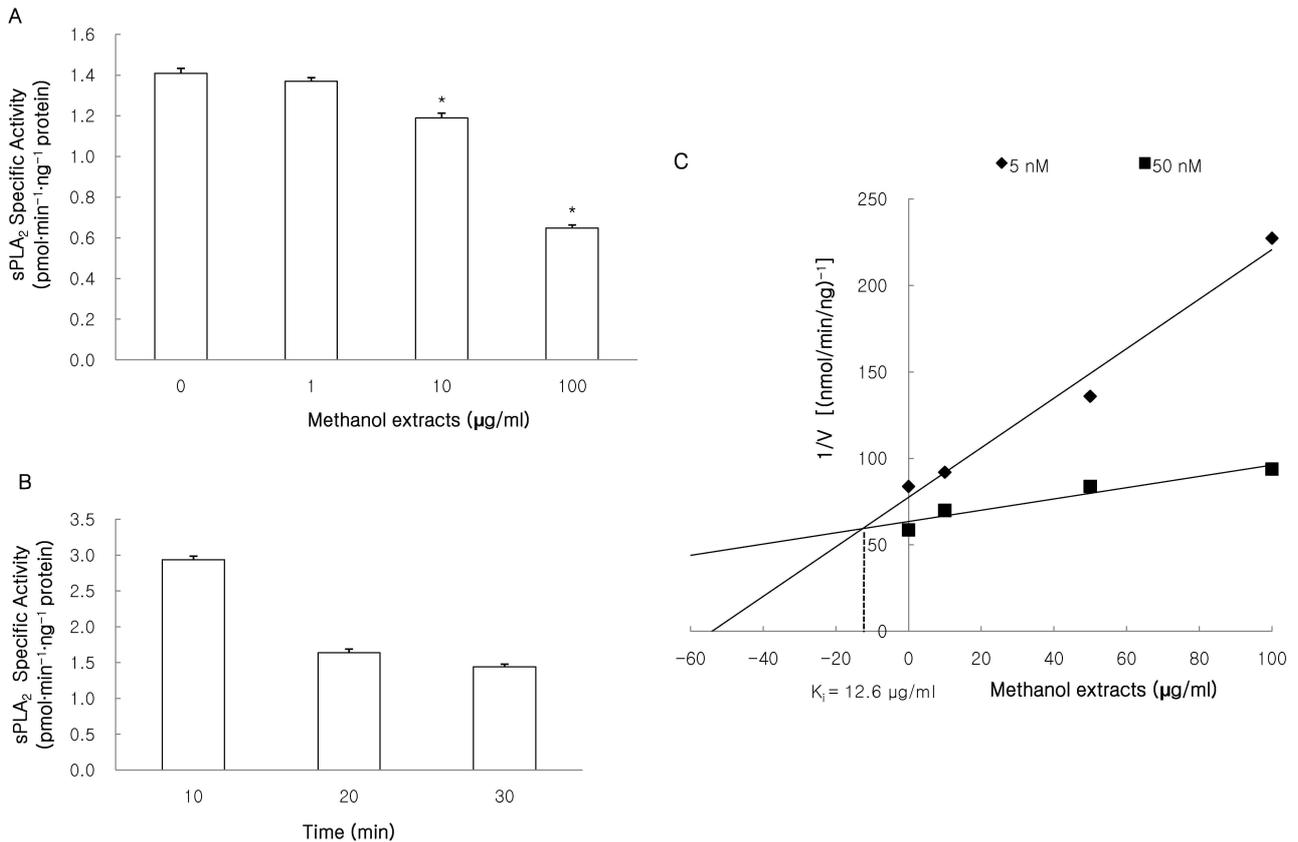
sPLA<sub>2</sub> obtained from honey bee venom hydrolyzed 10-pyrene PC; the sPLA<sub>2</sub>-specific substrate [18]. We used purified sPLA<sub>2</sub> from honey bee venom to investigate the inhibitory effects of the methanol extracts on sPLA<sub>2</sub>. The

methanol extracts inhibited sPLA<sub>2</sub> in a concentration- and time-dependent manner (Fig. 3A and 3B). The methanol extracts inhibited sPLA<sub>2</sub> activity by 15.6% and 53.9% at concentrations of 10 and 100  $\mu\text{g/ml}$ , respectively.

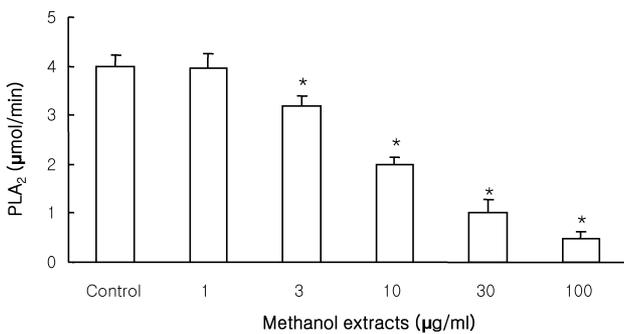
Dixon plots were constructed from sPLA<sub>2</sub>-induced hydrolysis rates of the substrate ([<sup>14</sup>C]AA-PC of 5 and 50 nM) to determine the sPLA<sub>2</sub> inhibitory patterns by the methanol extracts. Fig. 3C demonstrated that the apparent  $K_i$  value of the methanol extracts on sPLA<sub>2</sub> was 12.6  $\mu\text{g/ml}$ , and such inhibitory patterns suggest that methanol extracts acts as a competitive inhibitor against sPLA<sub>2</sub>. Additionally, the methanol extracts inhibited bee venom sPLA<sub>2</sub>-induced 10-pyrene PC hydrolysis in a concentration-dependent manner (Fig. 4).

## DISCUSSION

The methanol extracts decreased melittin-induced [<sup>3</sup>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<sub>2</sub> activator by increasing intracellular  $\text{Ca}^{2+}$  concentrations via receptor-operated calcium channels [19]. Such inhibitory effects of the methanol extracts may be associated with PLA<sub>2</sub> inhibition. However, AA release is an indirect measure of PLA<sub>2</sub> 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<sub>2</sub> activity. sPLA<sub>2</sub> activity was measured using 1-palmitoyl-2-[<sup>14</sup>C]arachidonyl phosphatidylcholine ([<sup>14</sup>C]AA-PC) as a substrate by previously described methods. The methanol extracts inhibited sPLA<sub>2</sub>-induced hydrolysis of [<sup>14</sup>C]AA-PC in a concentration (A)- and time (B)-dependent manner. A Dixon plot showed that the inhibition of sPLA<sub>2</sub> by methanol extracts appeared to be competitive with an inhibition constant (*K<sub>i</sub>*) 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<sub>2</sub> activity with 10-pyrene phosphatidylcholine (10-Pyrene PC). 10-Pyrene PC hydrolyzed by purified sPLA<sub>2</sub> 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<sub>2</sub> isozymes using [<sup>14</sup>C]AA-PC, a specific PLA<sub>2</sub> substrate to address this issue.

RAW 264.7 cellular lysates were used as the source of cPLA<sub>2</sub> since we did not prepare each PLA<sub>2</sub> isozyme. RAW

264.7 cell-derived PLA<sub>2</sub> was completely inhibited by AACOCF<sub>3</sub> but not by DTT or BEL. RAW cell-derived PLA<sub>2</sub> did not result in any PLA<sub>2</sub> activity using the sPLA<sub>2</sub>-specific substrate, 10-pyrene PC. Additionally, RAW 264.7 cell-derived PLA<sub>2</sub> showed only a low level of activity in the absence of Ca<sup>2+</sup> [17]. These data suggested that RAW 264.7 cell-derived PLA<sub>2</sub> primarily contained cPLA<sub>2</sub> rather than sPLA<sub>2</sub> and iPLA<sub>2</sub>.

We determined the inhibitory pattern of the methanol extracts on cPLA<sub>2</sub> with Dixon plots. The methanol extracts inhibited cPLA<sub>2</sub>-induced hydrolysis of [<sup>14</sup>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<sub>i</sub>*) of 3.7 µg/ml. It has been reported that cPLA<sub>2</sub> is inhibited by a trifluoromethyl ketone analog of AA (AACOCF<sub>3</sub>), which presumably binds directly to the active site of cPLA<sub>2</sub> [15,21,22]; AACOCF<sub>3</sub> (10 µM) inhibited cPLA<sub>2</sub> by 95.4%, and the methanol extracts (100 µg/ml) inhibited it by 63.2%. Bee venom sPLA<sub>2</sub> was used to investigate the effects of the methanol extracts on sPLA<sub>2</sub>. The methanol extracts inhibited sPLA<sub>2</sub>-induced hydrolysis of [<sup>14</sup>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<sub>i</sub>*)

of 12.6 µg/ml. These data suggest that methanol extracts of the *Morinda citrifolia* fruit inhibits both Ca<sup>2+</sup>-dependent PLA<sub>2</sub> such as cPLA<sub>2</sub> and sPLA<sub>2</sub>. *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<sup>2+</sup>-dependent PLA<sub>2</sub>.

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 [<sup>3</sup>H]AA release in RAW 264.7 cells in a concentration-dependent manner. The methanol extracts inhibited cPLA<sub>2</sub>/sPLA<sub>2</sub>-induced hydrolysis of [<sup>14</sup>C]AA-PC in both a concentration- and time-dependent manner. A Dixon plot demonstrated that cPLA<sub>2</sub> and sPLA<sub>2</sub> inhibition by the methanol extracts appeared to be competitive with an inhibition constant (*K<sub>i</sub>*) 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<sup>2+</sup>-dependent PLA<sub>2</sub>, such as cPLA<sub>2</sub> and sPLA<sub>2</sub>. These data supported that the anti-inflammatory activity of *Morinda citrifolia* can be secondary to the inhibition of Ca<sup>2+</sup>-dependent PLA<sub>2</sub>.

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