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*Article*

# Inhibition of Cytosolic Phospholipase $A_2\alpha$ (c$PLA_2\alpha$) by Medicinal Plants in Relation to Their Phenolic Content

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**Abstract:** The cytosolic phospholipase $A_2\alpha$ (c$PLA_2\alpha$) is one of the potential targets for anti-inflammatory drugs, since this enzyme plays a key role in the inflammation processes seen in health disorders, like asthma, allergic reactions, arthritis and neuronal diseases. In this study, c$PLA_2\alpha$ inhibition by 43 methanol extracts from medicinal plants rich in polyphenols was determined. The eight most active extracts were derived from *Ribes nigrum* ($IC_{50}$ of 27.7 µg/mL), *Ononis spinosa* ($IC_{50}$ of 39.4 µg/mL), *Urtica dioica* ($IC_{50}$ of 44.32 µg/mL), *Betula* sp. ($IC_{50}$ of 58.02 µg/mL), *Sanguisorba officinalis* ($IC_{50}$ of 76.25 µg/mL), *Orthosiphon stamineus* ($IC_{50}$ of 78.83 µg/mL), *Petasites hybridus* ($IC_{50}$ of 81.02 µg/mL) and *Tussilago farfara* ($IC_{50}$ of 123.28 µg/mL). Additionally, the antioxidant activities of these extracts were determined with the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and their phenolic content with the Folin–Ciocalteu reagent. Antioxidant activity showed a non-linear, positive correlation to the phenolic content, but no correlation of $PLA_2\alpha$ inhibition with phenolic content could be established. This study provides evidence that c$PLA_2\alpha$ may be a relevant target for anti-inflammatory agents.

**Keywords:** cytosolic phospholipase $A_2\alpha$; anti-inflammatory; arachidonic acid; medicinal plants; phenolic content; antioxidant activity; DPPH; Folin–Ciocalteu reagent

## 1. Introduction

The phospholipases $A_2$ ($PLA_2$) play an important role in the inflammatory response. The enzymes catalyze the release of a fatty acid at the $sn_2$-position of membrane phospholipids. This leads to the release of a lysophospholipid, which itself functions as a signal molecule, and arachidonic acid (AA), a poly-unsaturated fatty acid. AA is the precursor for the synthesis of the eicosanoids, which are lipid mediators of the inflammatory response. Two well-known subgroups of eicosanoids are the prostaglandins and leukotrienes, which are produced from AA through the enzymes cyclooxygenase 1 and 2 (COX-1 and COX-2) and 5-lipoxygenase (5-LOX), respectively [1]. Whereas COX enzymes are well-studied targets for anti-inflammatory drugs like acetylsalicylic acid, no drugs are on the market for 5-LOX and $PLA_2$, so far. Depending on their size, catalytic mechanism and specificity for AA, there are more than ten groups of $PLA_2$ enzymes [1]. Among $PLA_2$ enzymes, the cytosolic $PLA_2$ alpha from Subgroup IV ($cPLA_2\alpha$) is known to have the highest specificity for AA [2] and, therefore, is discussed as the key enzyme in the release of AA for eicosanoid synthesis [1,3]. $cPLA_2\alpha$ has been associated with inflammation-related diseases, like asthma and allergic reactions [4–6], arthritis [7,8] and neuronal diseases, such as Alzheimer's disease [9,10], multiple sclerosis [11] and Parkinson's disease [12]. Therefore, the $cPLA_2\alpha$ isoform should be a potential target for anti-inflammatory drugs [13].

In the history of the therapy of inflammation, medicinal plants have always played important roles [14]; still, their mechanisms of action are often unknown. In this study, porcine $cPLA_2\alpha$ inhibition by 43 methanol extracts of different medicinal plants was analyzed, known to accumulate potentially anti-inflammatory polyphenols, such as flavonoids, which are known to have an influence on the arachidonic acid metabolism [14–16]. Following this screening, we further examined the eight most potent inhibitory extracts in more detail. In addition, the total phenolic content of these extracts was quantified with Folin–Ciocalteu reagent and their antioxidant activity with DPPH. We further analyzed whether anti-inflammatory properties are correlated with phenolic content and anti-oxidant activities.

## 2. Results and Discussion

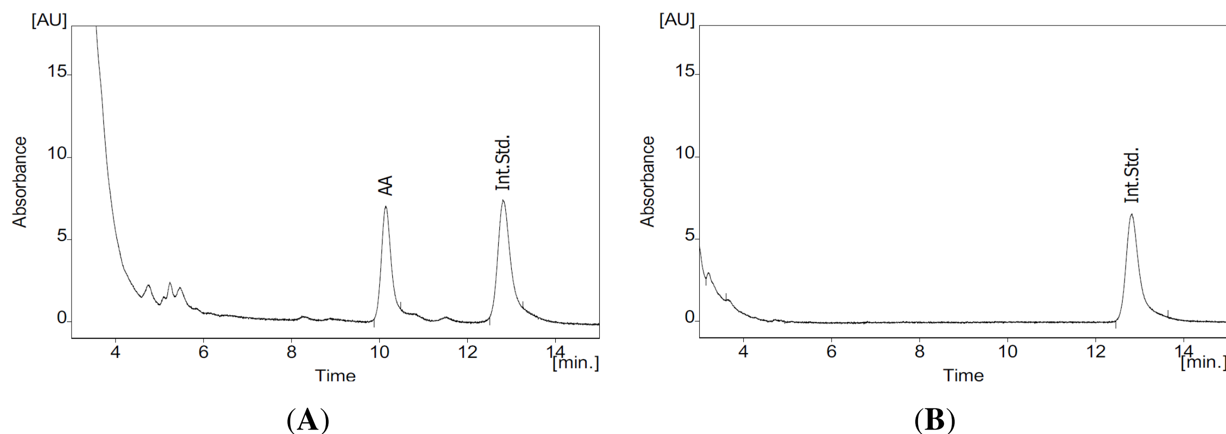
Initially, 43 methanol extracts from 43 species of 25 different plant families were screened for $cPLA_2\alpha$ inhibition at a final concentration of 100 µg/mL. As a positive control, the $cPLA_2\alpha$ inhibitor arachidonyl trifluoromethyl ketone (25 µM) was employed, resulting in an 80% inhibition. All experiments were conducted the same way, and no abnormalities occurred. Figure 1 shows an exemplary HPLC chromatogram from a sample of 100 µg/mL *Ononis spinosa* incubated with $cPLA_2\alpha$. Among the active extracts, 17 showed over 50% inhibition of AA release (see Table 1 for all screening results). From these, the eight best inhibitory extracts, *Urtica dioica* (leaf), *Petasites hybridus* (leaf), *Sanguisorba officinalis* (herb), *Ribes nigrum* (leaf), *Betula* sp. (leaf), *Ononis spinosa* (herb), *Orthosiphon stamineus* (leaf) and *Tussilago farfara* (leaf), in descending order, were chosen to establish the dose dependence of $cPLA_2\alpha$ inhibition.

The activity of $cPLA_2\alpha$ strongly depends on calcium ions, which are necessary for its binding to substrate [2,17]. Furthermore, enzymatic activity can be raised two to three times by phosphorylation [18,19]. It has been shown that phosphorylation of human $cPLA_2\alpha$ takes place within five minutes after stimulation of platelets with thrombin [18,20]. In our experimental setup, we isolate the porcine $cPLA_2\alpha$

from fresh blood. In the process of slaughtering, the pigs experience stress and injury, which may trigger the production of thrombin. Thus, during the process of platelet isolation from porcine blood in the lab, phosphorylation of porcine cPLA$_2\alpha$ may take place. However, we did not observe platelet coagulation as a consequence of thrombin production.

**Table 1.** cPLA$_2\alpha$ inhibition (%) by 100 µg/mL methanol extract of 43 plants. Species are ordered according to their inhibitory activity.

| Scientific Name | Plant Family | Part | Drug Name | Inhibition ± Error (%) |
|---|---|---|---|---|
| *Urtica dioica* L. | Urticaceae | leaf | Urticae folium | 74.7 ± 2.87 |
| *Petasites hybridus* (L.) GAERTN. | Asteraceae | leaf | Petasitidis folium | 73.01 ± 2.42 |
| *Sanguisorba officinalis* L. | Rosaceae | herb | Sanguisorbae herba | 70.24 ± 2.88 |
| *Ribes nigrum* L. | Grossulariaceae | leaf | Ribis nigri folium | 69.49 ± 8.49 |
| *Betula* sp. L. | Betulaceae | leaf | Betulae folium | 68.69 ± 13.3 |
| *Ononis spinosa* L. | Fabaceae | herb | Oninidis herba | 67.97 ± 7.94 |
| *Orthosiphon stamineus* BENTH | Lamiaceae | leaf | Orthosiphonis folium | 65.71 ± 7.64 |
| *Tussilago farfara* L. | Asteraceae | leaf | Farfarae folium | 54.91 ± 3.82 |
| *Paullinia cupana* KUNTH | Sapindaceae | fruit | Guaranae fructus | 54.71 ± 3.86 |
| *Senegalia catechu* (L.F.) HURTER & MABB. | Fabaceae | gum | Gummi catechu | 54.61 ± 7.2 |
| *Styphnolobium japonicum* (L.) SCHOTT | Fabaceae | flower | Sophorae flos | 53.68 ± 2.69 |
| *Helichrysum arenarium* (L.) MOENCH | Asteraceae | flower | Helichrysi flos | 53.12 ± 3.34 |
| *Hamamelis virginiana* L. | Hamamelidaceae | leaf | Hamamelidis folium | 52.82 ± 5.67 |
| *Camellia sinensis* (L.) KUNTZE | Theaceae | leaf | Theae folium | 52.46 ± 2.93 |
| *Centella asiatica* (L.) URBAN | Apiaceae | herb | Centellae herba | 51.85 ± 4.07 |
| *Tropaeolum majus* L. | Tropaeolaceae | herb | Tropaeoli herba | 50.85 ± 3.59 |
| *Arnica montana* L. | Asteraceae | flower | Arnicae flos | 50.06 ± 4.1 |
| *Tanacetum parthenium* (L.) SCH.BIP. | Asteraceae | herb | Tanaceti parthenii herba | 49.46 ± 3.92 |
| *Cynara cardunculus* L. | Asteraceae | leaf | Cynarae folium | 44.85 ± 4.52 |
| *Plantago lanceolata* L. | Plantaginaceae | leaf | Plantaginis lanceolatae folium | 42.25 ± 3.93 |
| *Leonurus cardiaca* L. | Lamiaceae | herb | Leonuri herba | 41.87 ± 3.79 |
| *Marsdenia cundurango* RCHB.F. | Apocynaceae | cortex | Condurango cortex | 41.84 ± 5.34 |
| *Melissa officinalis* L. | Lamiaceae | leaf | Melissae folium | 41.53 ± 4.48 |
| *Agathosma betulina* (BERG.) PILLANS | Rutaceae | leaf | Bucco folium | 39.49 ± 5.55 |
| *Solidago* sp. L. | Asteraceae | herb | Solidaginis herba | 36.66 ± 5.12 |
| *Quercus* sp. L. | Fagaceae | cortex | Quercus cortex | 36.29 ± 3.95 |
| *Salix alba* L. | Salicaceae | cortex | Salicis cortex | 34.33 ± 5.71 |
| *Crataegus* sp. TOURN. EX L. | Rosaceae | flower | Crataegi flos | 29.83 ± 18.31 |
| *Vaccinium vitis-idaea* L. | Ericaceae | leaf | Vitis idaei folium | 29.36 ± 6.88 |
| *Harungana madagascariensis* LAM. EX POIRET | Hypericaceae | cortex | Harongae cortex | 29.36 ± 4.16 |
| *Peumus boldus* MOLINA | Monimiaceae | leaf | boldo folium | 28.46 ± 8.37 |
| *Salvia officinalis* L. | Lamiaceae | leaf | Salviae folium | 26.16 ± 7.89 |
| *Berberis vulgaris* L. | Berberidaceae | cortex | Berberidis cortex | 24.9 ± 4.27 |
| *Juglans regia* L. | Juglandaceae | leaf | Juglandis folium | 23.88 ± 4.45 |
| *Arctostaphylos uva-ursi* (L.) SPRENG. | Ericaceae | leaf | Uvae-ursi folium | 22.04 ± 5.75 |
| *Verbascum* sp. L. | Scrophulariaceae | flower | Verbasci flos | 21.64 ± 4.49 |
| *Vaccinium myrtillus* L. | Ericaceae | leaf | Myrtilli folium | 19.17 ± 6.68 |
| *Filipendula ulmaria* (L.) MAXIM. | Rosaceae | herb | Filipendulae herba | 10.19 ± 10.43 |
| *Alchemilla vulgaris* L. | Rosaceae | herb | Alchemillae herba | 10.03 ± 5.76 |
| *Fragaria vesca* L. | Rosaceae | leaf | Fragariae folium | 7.28 ± 5.14 |
| *Hamamelis virginiana* L. | Hamamelidaceae | cortex | Hamamelidis cortex | 1.41 ± 12.42 |
| *Punica granatum* L. | Lythraceae | cortex | Granati cortex | −10.69 ± 6.81 |
| *Humulus lupulus* L. | Cannabaceae | glands | Lupuli glandula | −65.16 ± 10.73 |
| Arachidonyl Trifluoromethyl Ketone (25 µM) | - | - | - | 80.04 ± 3.58 |




(**A**) (**B**)

**Figure 1.** HPLC chromatogram of a sample of *Ononis spinosa* herb extract (100 µg/mL) incubated with cPLA$_2\alpha$. Absorbance (AU, y-axis) is depicted in relation to time (min, x-axis) at a detection wavelength of 200 nm (**A**) and 254 nm (**B**), respectively. AA = arachidonic acid peak; Int.Std. = the peak of the internal standard 4-undecyloxybenzoic acid.

### *2.1. Dose Dependence of cPLA$_2\alpha$ Inhibition*

All eight extracts exhibited a dose-dependent cPLA$_2\alpha$ inhibition (Figure 2). With an IC$_{50}$ value of 27.7 ± 4.71 µg/mL, the extract from *Ribes nigrum* was the most potent cPLA$_2\alpha$ inhibitor (Figure 2A), followed by *Ononis spinosa* (39.4 ± 6.49 µg/mL) (Figure 2B), *Urtica dioica* (44.32 ± 5.88 µg/mL) (Figure 2C) and *Betula* sp. (58.02 ± 5.99 µg/mL) (Figure 2D). A little less active were the extracts of *Sanguisorba officinalis* (Figure 2E), *Orthosiphon stamineus* (Figure 2F) and *Petasites hybridus* (Figure 2G), with corresponding IC$_{50}$ values of 76.25 ± 10.93 µg/mL, 78.83 ± 15.55 µg/mL and 81.02 ± 18.23 µg/mL, respectively. The least active extract in this selection came from *Tussilago farfara* with an IC$_{50}$ of 123.28 ± 15.06 µg/mL (Figure 2H).

Previous studies have reported anti-inflammatory activity for four of the eight plants, *Urtica dioica*, *Orthosiphon stamineus*, *Ribes nigrum* and *Ononis spinosa*, in the carragenan-induced paw edema model in rats [21–26]. The inflammatory reaction to carrageenan is biphasic: in the first phase, there is a release of histamine and serotonin, whereas the second phase is characterized by the release of eicosanoids, like prostaglandins [27–29]. This model can thus be used to study non-specific inflammation, including the release of arachidonic acid through cPLA$_2\alpha$. In addition to the effect in the paw edema model, *Urtica dioica* extracts were shown to inhibit COX-1 and COX-2 directly and to act as an antagonist at the histamine 1 receptor, a key target for allergic reactions [30,31]. With regard to the role of cPLA$_2\alpha$ enzyme in allergic reactions [4–6], the cPLA$_2\alpha$ inhibiting the potential of *Urtica dioica* may be important as an anti-allergy agent.

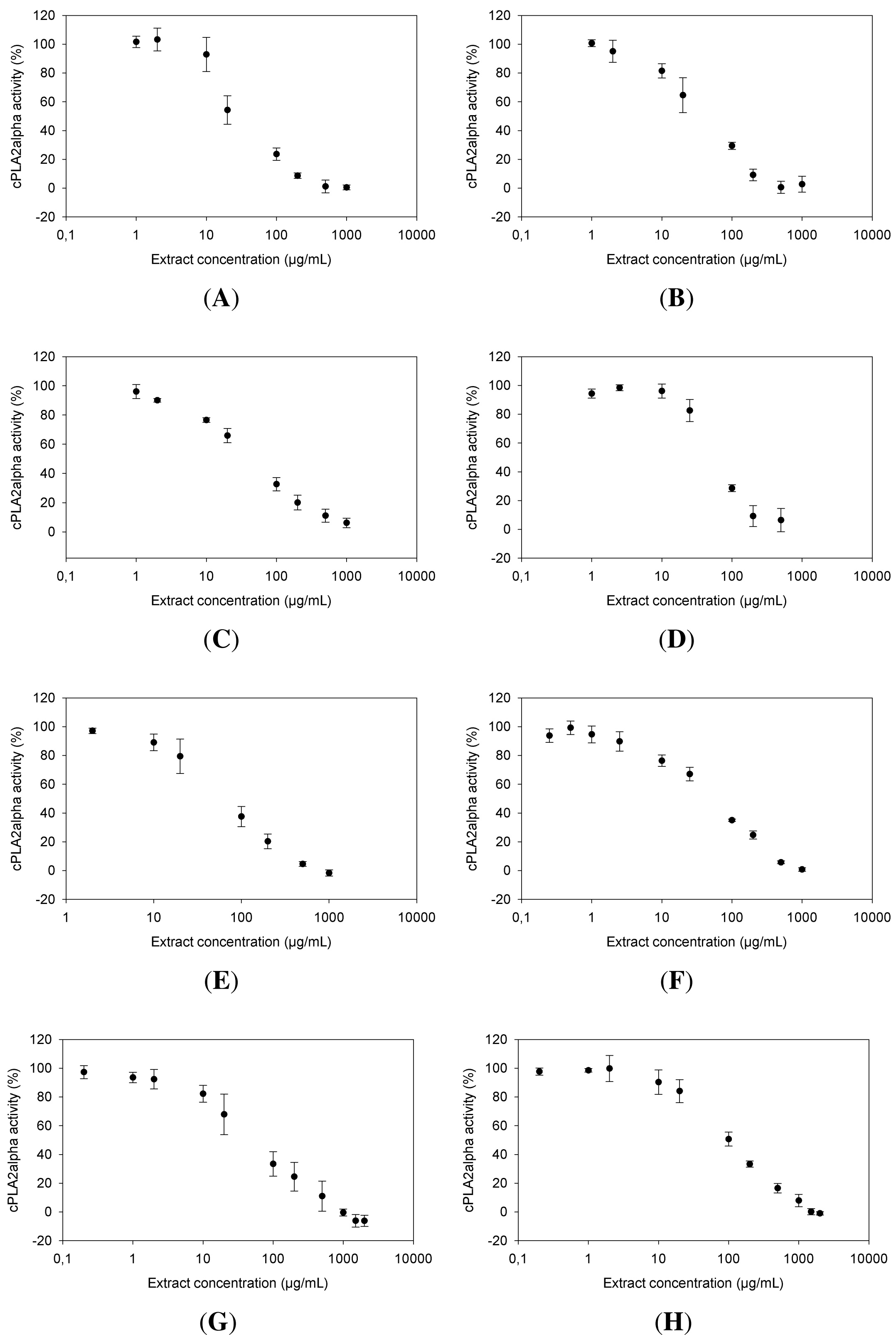



**Figure 2.** Dose-dependence of cytosolic phospholipase $A_2\alpha$ ($cPLA_2\alpha$) inhibition by extracts of: *Ribes nigrum* (**A**); *Ononis spinosa* (**B**); *Urtica dioica* (**C**); *Betula* sp. (**D**); *Sanguisorba officinalis* (**E**); *Orthosiphon stamineus* (**F**); *Petasites hybridus* (**G**); and *Tussilago farfara* (**H**). $cPLA_2\alpha$ activity (%) is plotted against extract concentration (µg/mL). The x-axes are scaled logarithmically.

As concerns *Sanguisorba officinalis* and *Ononis spinosa*, the roots are well-known traditional phytomedicines [14]. For the aerial parts of these plants, anti-inflammatory activities have not been reported. Root extracts of *Sanguisorba officinalis* exhibited an anti-asthmatic and anti-allergic effect in mouse models [32,33]. *In vitro* assays with lipopolysaccharide and interferon gamma stimulated mouse macrophages showed the potential of a *Sanguisorba officinalis* extract to substantially inhibit prostaglandin, nitric oxide (NO) and tumor necrosis factor alpha production [34,35]. *Ononis spinosa* root extract showed an analgesic effect in mice equivalent to the positive control aspirin [36] and was able to reduce carrageenan-induced paw edema in rats [21]. We now provide evidence that cPLA$_2\alpha$ is probably the target for the anti-inflammatory activity of the aerial parts from *Sanguisorba officinalis* and *Ononis spinosa*.

Prostaglandin and leukotriene synthesis were inhibited by extracts from *Petasites hybridus* [37,38]. Furthermore, extracts from this plant are marketed as a prophylactic for the treatment of migraine [39,40]. However, its use in the therapy of allergic rhinitis and asthma has been discussed controversially [41–44]. In this study, we provide evidence that *Petasites hybridus* directly inhibits cPLA$_2\alpha$, which would also explain the reported inflammatory and anti-allergic activities. The extract of *Tussilago farfara* and its ingredient tussilagon inhibit NO and prostaglandin production in stimulated murine microglia cells and macrophages [35,45,46]. The anti-inflammatory effect of tussilagone may be due to an induction of heme oxygenase-1 [47] and an inhibition of COX-2 and nitric oxide synthase gene expression [45]. The anti-inflammatory activity is moderate, as seen from our results of cPLA$_2\alpha$ inhibition.

In traditional medicine, *Betula sp.* leaves are used to treat inflammation [14]. This may be the result of a variety of flavonoids and triterpenoids present [48–50]. *Betula pendula* leaf extract showed an inhibitory effect against corneal inflammation in rats [51], lymphocyte growth and cell division [52]. Our results indicate that the anti-inflammatory effect of *Betula sp.* may involve the inhibition of cPLA$_2\alpha$.

### *2.2. cPLA$_2\alpha$ Inhibition in Relation to Phenolic Content and Antioxidant Activities*

Phenolic secondary metabolites, for example flavonoids, have the ability to unspecifically interact with all kinds of biomolecules, especially proteins. This results, for example, in an inhibition of the catalytic mechanism of enzymes [53]. As a consequence, polyphenols could directly inhibit cPLA$_2\alpha$, which would lead to a lower AA release. As expected, the results of the Folin–Ciocalteu assay confirmed that all eight extracts contain phenolic ingredients (see Table 2). It is very interesting that four of the plants—*Betula* sp., *Orthosiphon stamineus*, *Urtica dioica* and *Ononis spinosa*—are used as diuretics in phytomedicine [14]. The diuretic effect is attributed to the flavonoid content of these plants. Many flavonoids are known for their anti-inflammatory properties [15,49,54].

The Folin–Ciocalteu reagent not only reacts to phenolic compounds, but also to reducing agents [55]. To evaluate this interference, the reducing power of the extracts was tested with the stable free radical DPPH. As a control, the $EC_{50}$ value of ascorbic acid was determined and was expectedly low with $3.18 \pm 0.15$ µg/mL [56]. Several previous studies confirmed a correlation between the phenolic content determined with Folin–Ciocalteu reagent and the reducing capacity of different plant extracts [57–60]. In view of the mechanism, which in both assays is based on electron transfer, this is no surprise [55].

**Table 2.** Summary of cPLA$_2\alpha$ inhibition, phenolic content and DPPH scavenging activity of the eight most potent extracts. The cPLA$_2\alpha$ inhibition is shown as the IC$_{50}$ value (μg/mL final extract concentration); phenolic content relates to gallic acid equivalents (GAE) in mg per g extract. The DPPH radical scavenging is shown as EC$_{50}$ (μg/mL final extract concentration). Values are means ($n$ = 3) ± the standard error (SE), ordered according to their cPLA$_2\alpha$ inhibitory activity.

| Extract | cPLA$_2\alpha$ Inhibition (IC$_{50}$) μg/mL ± SE | Phenolic Content (GAE) mg/g ± SE | Radical Scavenging (EC$_{50}$) μg/mL ± SE |
|---|---|---|---|
| *Ribes nigrum* | 27.7 ± 4.71 | 131.25 ± 7.15 | 13.36 ± 0.6 |
| *Ononis spinosa* | 39.4 ± 6.49 | 20.55 ± 2.56 | 271.07 ± 13.13 |
| *Urtica dioica* | 44.32 ± 5.88 | 38.26 ± 2.41 | 90.5 ± 4.01 |
| *Betula* sp. | 58.02 ± 5.99 | 62.59 ± 2.38 | 27.17 ± 1.26 |
| *Sanguisorba officinalis* | 76.25 ± 10.93 | 116.96 ± 5.89 | 14.93 ± 0.66 |
| *Orthosiphon stamineus* | 78.83 ± 15.55 | 50.2 ± 0.26 | 30.33 ± 2.06 |
| *Petasites hybridus* | 81.02 ± 18.23 | 122.61 ± 4.73 | 14.27 ± 0.76 |
| *Tussilago farfara* | 123.28 ± 15.06 | 122.39 ± 5.46 | 14.54 ± 0.72 |

EC$_{50}$ values for DPPH reduction range between 13.36 ± 0.6 μg/mL (*Ribes nigrum*) and 271.07 ± 13.13 μg/mL (*Ononis spinosa*). With an IC$_{50}$ value of >200 μg/mL, *Ononis spinosa* may be considered as not active. When our results of the radical scavenging are plotted against the phenolic content, an inverse non-linear correlation can be seen (see Table 2, Columns 2 and 3, and Figure 3A), meaning that the higher the phenolic content, the stronger the antioxidant effects. Hence, the extracts with higher phenolic content also show higher radical scavenging activity (evident in a low EC$_{50}$).

While radical scavenging activity and phenolic content correlate very well, cPLA$_2\alpha$ inhibition is apparently not correlated with the phenolic content (Figure 3B). The best example is *Tussilago farfara* extract, which showed the lowest cPLA$_2\alpha$ inhibition by far, combined with the third highest phenolic content. However, *Ribes nigrum* extract showed the highest phenolic content together with the strongest cPLA$_2\alpha$ inhibition (Table 2).

### 2.3. *Urtica dioica and Ononis spinosa as Promising New Anti-Inflammatory Drugs with Regard to cPLA$_2\alpha$ as a Target*

Polyphenols unspecifically bind to proteins, thereby often resulting in conformational changes that lead to inhibition of the enzymatic activity [53]. However, phenolic secondary metabolites may not be the main cause for cPLA$_2\alpha$ inhibition, since there seems to be no correlation between phenolic content of the eight methanol extracts and their cPLA$_2\alpha$ inhibition. Furthermore, some polyphenol-rich plant extracts, such as *Punica granatum* and *Humulus lupulus*, did not inhibit cPLA$_2\alpha$, but instead appear to act as agonists, as the AA production was elevated to 110% and 165%, compared to the control (see Table 1). Therefore, other non-phenolic ingredients may be responsible for cPLA$_2\alpha$ inhibition. Two extracts showing very low phenolic content, but high cPLA$_2\alpha$ inhibition, are *Ononis spinosa* and *Urtica dioica*.

Since these two extracts seem to be the most promising cPLA$_2\alpha$ inhibitors despite their low phenolic content, we assume that they may contain specific cPLA$_2\alpha$ inhibitors.

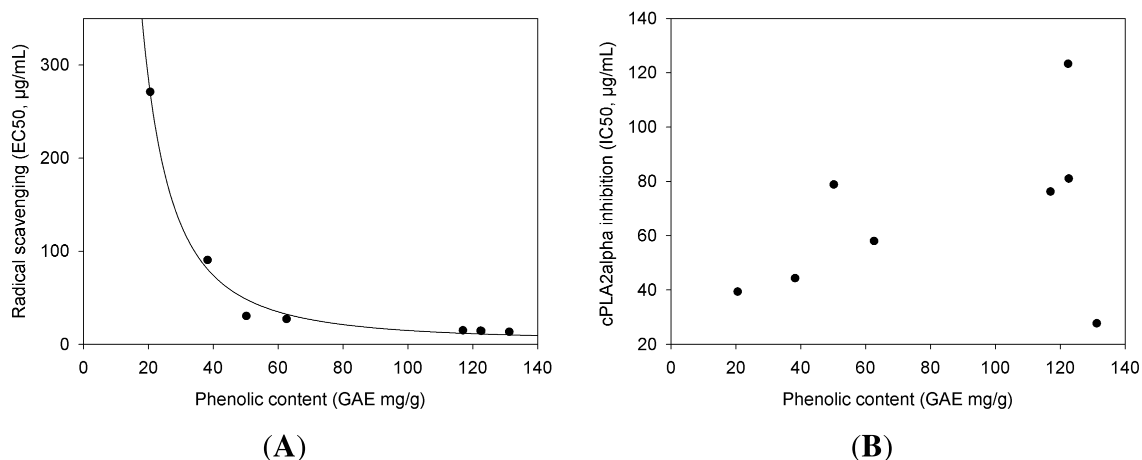



**Figure 3.** Correlation of antioxidant activities and cPLA$_2\alpha$ inhibition with the phenolic content of the extracts. In (**A**), the EC$_{50}$ values of radical scavenging (µg/mL extract concentration, y-axis) are plotted against the phenolic content (GAE mg/g, x-axis). The non-linear regression curve is calculated with SigmaPlot (polynomial, inverse second order, simplified), resulting in the following equation: $DPPH\ scavenging = 3.6 + \frac{1.1 \cdot 10^5}{phenolic\ content^2}$; (**B**) The IC$_{50}$ values of cPLA$_2\alpha$ inhibition (µg/mL extract concentration, y-axis) plotted against the phenolic content (GAE mg/g, x-axis). The values do not reveal a correlation. (**A**): DPPH (EC$_{50}$) *vs.* phenolic content; (**B**): cPLA$_2\alpha$ inh. (IC$_{50}$) *vs.* phenolic content.

## 3. Experimental Section

### *3.1. Reagents and Chemicals*

HPLC-grade water and acetonitrile, nordihydroguaiaretic acid (NDGA), 4-undecyloxybenzoic acid, Folin–Ciocalteu phenol reagent (2 N), protease inhibitor cocktail (containing AEBSF, aprotinin, Bestatin, E-64, leupeptin and pepstatin A) were purchased from Sigma-Aldrich (Taufkirchen, Germany); 1-stearoyl-2-arachidonoyl-phosphatidylcholine (SAPC), 1,2-dioleoyl-*sn*-glycerol (DOG) and bromoenol lactone were from Cayman Chemical (Ann Arbor, MI, USA, delivered via Biomol, Hamburg, Germany); dithiothreitol (DTT) from Applichem (Darmstadt, Germany) and ascorbic acid were from Carl Roth (Karlsruhe, Germany).

### *3.2. Isolation of cPLA$_2\alpha$ from Porcine Platelets*

Fresh porcine blood (750 mL) from a nearby slaughterhouse (Fleischversorgungszentrum, Mannheim, Germany) was immediately mixed 1:5 with citrate buffer (85 mM $Na_3$Citrate × 2 $H_2O$, 66.6 mM citric acid and 111 mM D(+)glucose) and subsequently centrifuged at portions of 20 mL (per 50 mL tube) at 2000× *g* (Hettich Rotina 380R, Hettich Lab Technology, Tuttlingen, Germany) for 3 min at 4 °C.

The platelet-rich supernatant was transferred to fresh tubes, and platelets were centrifuged at 1600× *g* for 20 min at 4 °C. The pellet was resuspended with 3 mM EDTA in phosphate-buffered saline (137 mM NaCl, 2.68 mM KCl, 10.14 mM $Na_2HPO_4$, 1.76 mM $KH_2PO_4$, pH 7.5) and centrifuged again. Platelet lysis and cPLA$_2\alpha$ isolation were conducted as described in [61], with minor changes. Briefly, platelets were lysed in a hypotonic buffer at −20 °C over night after the addition of protease inhibitor cocktail. After sonification (Omni-Ruptor 4000, Omni International Inc., Kennesaw, GA, USA, 2 times 30 s, 50% intensity), cell debris was separated by centrifugation at 48,000× *g* for 60 min at 4 °C (Beckman J2-21 centrifuge, rotor type JA-20, Beckman Coulter, Krefeld, Germany). The clear supernatant was diluted 1:1 with Buffer A (25 mM Tris, 1 mM EGTA, 2 mM DTT, pH 8.0) and transferred to a HiPrep™ Q XL 16/10 anionic exchange column (GE Healthcare Europe, Freiburg, Germany, column volume: 20 mL, flow rate: 5 mL/min), which was conditioned with the subsequent addition of 100 mL Buffer A, 100 mL Buffer B (1 M NaCl, 25 mM Tris, 1 mM EGTA, 2 mM DTT, pH 8.0) and 140 mL Buffer A again. After sample loading, the column was washed with 100 mL Buffer A, following a stepwise elution of 4 fractions of 60 mL each. The fractions were eluted with different NaCl concentrations in buffer (realized through a mixture of Buffers A and B), which were 150, 300, 450 and 600 mM each. The 450 mM NaCl fraction contained the cPLA$_2\alpha$ and was concentrated through a centrifugal filter with a molecular weight cut-off of 50 kDa (Vivaspin® 20, Sartorius, Göttingen, Germany) at 4000× *g* (4 °C). The resulting volume of 7 mL was incubated for 10 min at room temperature with bromoenol lactone (5 µM final concentration) to permanently inhibit the activity of the calcium-independent PLA$_2$ isoform. Secreted PLA$_2$ (sPLA$_2$) isoforms have up to eight disulfide bonds essential for their activity. By adding DTT to Buffer A and to the assay buffer, these disulfide bonds get reduced to free thiols, resulting in an activity loss of the sPLA$_2$ isoforms [1,2,62]. In contrast to the sPLA$_2$s, cPLA$_2\alpha$ is only active without disulfide bonds in the reducing environment of the cytosol, which is mimicked by the DTT [62,63]. Other cPLA$_2$ isoforms are considered negligible, since arachidonyl trifluoromethyl ketone, a specific cPLA$_2\alpha$ inhibitor [64], inhibits arachidonic acid production over 96% in our test system (at a concentration of 200 µM). The protein solution was diluted in Buffer A to liberate about 1 nmol AA in 60 min incubation at 37 °C. The enzyme fraction was stored in aliquots at −80 °C.

### *3.3. Incubation Procedure for the cPLA$_2\alpha$ Assay*

The incubation procedure and solid phase extraction was executed as described in [61], with some modifications. In short, 2 µL of extract (or dimethyl sulfoxide (DMSO) in the case of the control and blank) were incubated together with 10 µL isolated enzyme (blank: 10 µL buffer) and 88 µL mixed micelle emulsion as a substrate (formed by 200 µM SAPC and 100 µM DOG). After a 60-min incubation time at 37 °C, the reaction was stopped by adding 200 µL of acetonitrile/methanol/$Na_2$EDTA (16:15:1) containing nordihydroguaiaretic acid (NDGA, 0.6 µg/200 µL) and 4-undecyloxybenzoic acid as the internal standard (156 ng/200 µL). After solid-phase extraction according to [65] with BondElut C18 columns (100 mg sorbent, 3 mL volume; Agilent technologies, Santa Clara, CA, USA), the eluate was diluted 1:1 with water for HPLC analysis.

The initial PLA$_2\alpha$ inhibition screening was conducted with 43 methanol extracts (final concentration: 100 µg/mL), once in duplets. To establish dose dependence, the eight most potent inhibitors were tested

in different concentrations in duplets in three independent experiments ($n$ = 3). Controls and blanks were carried out in triplets for all experiments.

### *3.4. HPLC Analysis of AA*

For quantification of AA, a Young Lin YL9100 HPLC system (Young Lin Instrument Co. Ltd., Anyang, Korea, quaternary pump, degasser and diode array detector) connected to a Spark Marathon autosampler (Spark Holland, Emmen, The Netherlands) with a 200 µL sample loop was used. Using an isocratic program described in [65] with acetonitrile/water/phosphoric acid (77:23:0.1 *v*/*v*/*v*) as the mobile phase, 200 µL of the prepared sample was injected, and chromatographic separation was carried out with a Nucleosil C18 column (Macherey-Nagel, Düren, Germany, 3 mm inner diameter, 3 µm particle size, 125 mm length). AA and internal standard were detected at a wavelengths of 200 nm and 254 nm, respectively. For quantification of the relative enzyme activity, the AA peak area was set in relation to the internal standard peak area. The control (2 µL DMSO, 10 µL enzyme, 88 µL substrate) was defined as 100% activity, whereas the blank (2 µL DMSO, 10 µL buffer and 88 µL substrate) was defined as 0% activity.

### *3.5. Quantification of the Phenolic Agents with Folin–Ciocalteu Reagent*

This assay was used to quantify the phenolic content of the extracts. It is an adapted method from [66]. From each extract, 20 µL of a dilution in DMSO (1 mg/mL) was transferred in triplets to a 96-well plate. After addition of the Folin–Ciocalteu reagent (100 µL), the mixture was alkalinized with 80 µL of $Na_2CO_3$ solution (7.5% (*w*/*v*) in HPLC-grade water). For calibration, a dilution series of gallic acid (0 to 1.25 mg/mL) was treated the same way. Subsequently, the 96-well plate was incubated in the dark at room temperature for 60 min, following the measurement of absorption at 750 nm with a plate reader (Tecan Infinite M200 Pro, Tecan Group Ltd., Männedorf, Switzerland). To minimize the influence of inherent absorption from the extracts, the absorption of blanks containing 100 µL water instead of Folin–Ciocalteu reagent were subtracted from the absorptions of the samples. The same was done for the gallic acid standards. Using gallic acid, a calibration curve was established, and the phenolic content of the extracts was calculated using gallic acid equivalents (GAE), quantified as mg of gallic acid per g of extract.

### *3.6. DPPH Assay*

For evaluation of the reducing potential, the DPPH radical scavenging activity was determined. This assay was originally established in [67]. One hundred microliters of diluted extract were transferred to a 96-well plate in duplicates. Subsequently, 100 µL of DPPH solution (0.2 mM in methanol) were added, and incubation took place for 30 min in the dark at room temperature, following the determination of the absorption at 517 nm (Tecan Infinite M200 Pro). Final concentrations of the extracts ranged between 0.3 µg/mL to 5 mg/mL. As a control, 100 µL methanol was mixed with 100 µL of DPPH solution. The inherent absorption of the extracts was measured separately (extract and methanol) and

subtracted from each sample's absorption. Ascorbic acid (0.06 μg/mL to 62.5 μg/mL) served as a standard control. Radical scavenging was calculated with the following formula:

$$scavenging(\%) = 100 \cdot \frac{A_0 - A_s}{A_0}$$

with $A_0$ = control (DPPH + methanol) and $A_s$ = sample (DPPH + extract or ascorbic acid, respectively).

### *3.7. Statistics*

For the $PLA_2\alpha$ inhibition screening, errors were calculated with Gaussian error propagation. Standard deviations from the mean values were calculated for the dose-dependent studies and phenolic content. The $IC_{50}$ and $EC_{50}$ concentrations and their standard errors were calculated with SigmaPlot 11.0 (standard curve analysis with four parameter logistic).

## 4. Conclusions

In this study, we have provided evidence that polyphenol-rich medicinal plants often exert anti-inflammatory activities, which may be due to an inhibition of $cPLA_2\alpha$, which has been rather neglected as a relevant target for anti-inflammatory drugs. In another series of experiments, we have tried to identify the active substances in these extracts (Arnold and Wink, in preparation). However, possible synergistic effects from two or more of the ingredients in the extracts should not be left out of consideration [53].

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## Author Contributions

$cPLA_2\alpha$ inhibition experiments were carried out by E.A., T.B. and C.Z.; determination of phenolic content and DPPH radical scavenging activity by E.A. Experiments were designed by E.A. and M.W. The manuscript was written by E.A. and revised by M.W.

## Conflicts of Interest

The authors declare no conflict of interest.

## References

1. Dennis, E.A.; Cao, J.; Hsu, Y.H.; Magrioti, V.; Kokotos, G. Phospholipase $A_2$ enzymes: Physical structure, biological function, disease implication, chemical inhibition, and therapeutic intervention. *Chem. Rev.* **2011**, *111*, 6130–6185.
2. Clark, J.D.; Lin, L.L.; Kriz, R.W.; Ramesha, C.S.; Sultzman, L.A.; Lin, A.Y.; Milona, N.; Knopf, J.L. A novel arachidonic acid-selective cytosolic $PLA_2$ contains a $Ca^{2+}$-dependent translocation domain with homology to PKC and GAP. *Cell* **1991**, *65*, 1043–1051.
3. Balsinde, J.; Winstead, M.V.; Dennis, E.A. Phospholipase $A_2$ regulation of arachidonic acid mobilization. *FEBS Lett.* **2002**, *531*, 2–6.
4. Sokolowska, M.; Stefanska, J.; Wodz-Naskiewicz, K.; Cieslak, M.; Pawliczak, R. Cytosolic phospholipase $A_2$ group IVA is overexpressed in patients with persistent asthma and regulated by the promoter microsatellites. *J. Allergy Clin. Immunol.* **2010**, *125*, 1393–1395.
5. Mruwat, R.; Yedgar, S.; Lavon, I.; Ariel, A.; Krimsky, M.; Shoseyov, D. Phospholipase $A_2$ in experimental allergic bronchitis: A lesson from mouse and rat models. *PLoS ONE* **2013**, *8*, e76641.
6. Pniewska, E.; Sokolowska, M.; Kupryś-Lipińska, I.; Przybek, M.; Kuna, P.; Pawliczak, R. The step further to understand the role of cytosolic phospholipase $A_2$ Alpha and group X secretory phospholipase $A_2$ in allergic inflammation: Pilot study. *Biomed. Res. Int.* **2014**, *2014*, 670814, doi:10.1155/2014/670814.
7. Sommerfelt, R.M.; Feuerherm, A.J.; Jones, K.; Johansen, B. Cytosolic phospholipase A2 regulates TNF-induced production of joint destructive effectors in synoviocytes. *PLoS ONE* **2013**, *8*, e83555.
8. Kokotos, G.; Feuerherm, A.J.; Barbayianni, E.; Shah, I.; Sæther, M.; Magrioti, V.; Nguyen, T.; Constantinou-Kokotou, V.; Dennis, E.A.; Johansen, B. Inhibition of group IVA cytosolic phospholipase $A_2$ by thiazolyl ketones *in vitro*, *ex vivo*, and *in vivo*. *J. Med. Chem.* **2014**, *57*, 7523–7535.
9. Stephenson, D.; Rash, K.; Smalstig, B.; Roberts, E.; Johnstone, E.; Sharp, J.; Panetta, J.; Little, S.; Kramer, R.; Clemens, J. Cytosolic phospholipase $A_2$ is induced in reactive glia following different forms of neurodegeneration. *Glia* **1999**, *27*, 110–128.
10. Sagy-Bross, C.; Kasianov, K.; Solomonov, Y.; Braiman, A.; Friedman, A.; Hadad, N.; Levy, R. The role of Cytosolic Phospholipase $A_2\alpha$ in Amyloid Precursor Protein induction by amyloid $beta_{1-42}$-Implication for Neurodegeneration. *J. Neurochem.* **2014**, *132*, 559–571.
11. Kalyvas, A.; David, S. Cytosolic phospholipase $A_2$ plays a key role in the pathogenesis of multiple sclerosis-like disease. *Neuron* **2004**, *41*, 323–335.
12. Lee, H.J.; Bazinet, R.P.; Rapoport, S.I.; Bhattacharjee, A.K. Brain arachidonic acid cascade enzymes are upregulated in a rat model of unilateral Parkinson disease. *Neurochem. Res.* **2010**, *35*, 613–619.
13. Meyer, M.C.; Rastogi, P.; Beckett, C.S.; McHowat, J. Phospholipase $A_2$ inhibitors as potential anti-inflammatory agents. *Curr. Pharm. Des.* **2005**, *11*, 1301–1312.
14. Van Wyk, B.E.; Wink, M. *Medicinal Plants of the World: An Illustrated Scientific Guide to Important Medicinal Plants and Their Uses*, 2nd ed.; Timber Press: Portland, OR, USA, 2004.

15. Serafini, M.; Peluso, I.; Raguzzini, A. Flavonoids as anti-inflammatory agents. *Proc. Nutr. Soc.* **2010**, *69*, 273–278.
16. Ferrándiz, M.L.; Alcaraz, M.J. Anti-inflammatory activity and inhibition of arachidonic acid metabolism by flavonoids. *Agents Actions* **1991**, *32*, 283–288.
17. Channon, J.Y.; Leslie, C.C. A calcium-dependent mechanism for associating a soluble arachidonoyl-hydrolyzing phospholipase $A_2$ with membrane in the macrophage cell line RAW 264.7. *J. Biol. Chem.* **1990**, *265*, 5409–5413.
18. Kramer, R.M.; Roberts, E.F.; Manetta, J.V.; Hyslop, P.A.; Jakubowski, J.A. Thrombin-induced phosphorylation and activation of $Ca^{2+}$-sensitive cytosolic phospholipase $A_2$ in human platelets. *J. Biol. Chem.* **1993**, *268*, 26796–26804.
19. Lin, L.L.; Wartmann, M.; Lin, A.Y.; Knopf, J.L.; Seth, A.; Davis, R.J. $cPLA_2$ is phosphorylated and activated by MAP kinase. *Cell* **1993**, *72*, 269–278.
20. Kramer, R.M.; Roberts, E.F.; Strifler, B.A.; Johnstone, E.M. Thrombin induces activation of p38 MAP kinase in human platelets. *J. Biol. Chem.* **1995**, *270*, 27395–27398.
21. Bolle, P.; Faccendini, P.; Bello, U.; Panzironi, C.; Tita, B. *Ononis spinosa* L.: Pharmacological effect of ethanol extract. *Pharmacol. Res.* **1993**, *27*, 27–28.
22. Garbacki, N.; Tits, M.; Angenot, L.; Damas, J. Inhibitory effects of proanthocyanidins from *Ribes nigrum* leaves on carrageenin acute inflammatory reactions induced in rats. *BMC Pharmacol.* **2004**, *4*, doi:10.1186/1471-2210-4-25.
23. Dar, S.A.; Ganai, F.A.; Yousuf, A.R.; Balkhi, M.U.H.; Bhat, T.M.; Sharma, P. Pharmacological and toxicological evaluation of *Urtica dioica*. *Pharm. Biol.* **2013**, *51*, 170–180.
24. Declume, C. Anti-inflammatory evaluation of a hydroalcoholic extract of black currant leaves (*Ribes nigrum*). *J. Ethnopharmacol.* **1989**, *27*, 91–98.
25. Hajhashemi, V.; Klooshani, V. Antinociceptive and anti-inflammatory effects of *Urtica dioica* leaf extract in animal models. *Avicenna J. Phytomedicine* **2013**, *3*, 193–200.
26. Yam, M.F.; Lim, V.; Salman, I.M.; Ameer, O.Z.; Ang, L.F.; Rosidah, N.; Abdulkarim, M.F.; Abdullah, G.Z.; Basir, R.; Sadikun, A.; *et al.* HPLC and anti-inflammatory studies of the flavonoid rich chloroform extract fraction of *Orthosiphon stamineus* leaves. *Molecules* **2010**, *15*, 4452–4466.
27. Di Rosa, M.; Giroud, J.P.; Willoughby, D.A. Studies on the mediators of the acute inflammatory response induced in rats in different sites by carrageenan and turpentine. *J. Pathol.* **1971**, *104*, 15–29.
28. Di Rosa, M. Biological properties of carrageenan. *J. Pharm. Pharmacol.* **1972**, *24*, 89–102.
29. Vinegar, R.; Truax, J.F.; Selph, J.L.; Johnston, P.R.; Venable, A.L.; McKenzie, K.K. Pathway to carrageenan-induced inflammation in the hind limb of the rat. *Fed. Proc.* **1987**, *46*, 118–126.
30. Farag, M.A.; Weigend, M.; Luebert, F.; Brokamp, G.; Wessjohann, L.A. Phytochemical, phylogenetic, and anti-inflammatory evaluation of 43 *Urtica* accessions (stinging nettle) based on UPLC-Q-TOF-MS metabolomic profiles. *Phytochemistry* **2013**, *96*, 170–183.
31. Roschek, B., Jr.; Fink, R.C.; McMichael, M.; Alberte, R.S. Nettle extract (*Urtica dioica*) affects key receptors and enzymes associated with allergic rhinitis. *Phytother. Res.* **2009**, *23*, 920–926.
32. Lee, N.H.; Lee, M.Y.; Lee, J.A.; Jung, D.Y.; Seo, C.S.; Kim, J.H.; Shin, H.K. Anti-asthmatic effect of *Sanguisorba officinalis* L. and potential role of heme oxygenase-1 in an ovalbumin-induced murine asthma model. *Int. J. Mol. Med.* **2010**, *26*, 201–208.

33. Shin, T.Y.; Lee, K.B.; Kim, S.H. Anti-allergic effects of Sanguisorba officinalis on animal models of allergic reactions. *Immunopharmacol. Immunotoxicol.* **2002**, *24*, 455–468.
34. Yu, T.; Lee, Y.J.; Yang, H.M.; Han, S.; Kim, J.H.; Lee, Y.; Kim, C.; Han, M.H.; Kim, M.Y.; Lee, J.; *et al.* Inhibitory effect of *Sanguisorba officinalis* ethanol extract on NO and $PGE_2$ production is mediated by suppression of NF-κB and AP-1 activation signaling cascade. *J. Ethnopharmacol.* **2011**, *134*, 11–17.
35. Ravipati, A.S.; Zhang, L.; Koyyalamudi, S.R.; Jeong, S.C.; Reddy, N.; Bartlett, J.; Smith, P.T.; Shanmugam, K.; Münch, G.; Wu, M.J.; *et al.* Antioxidant and anti-inflammatory activities of selected Chinese medicinal plants and their relation with antioxidant content. *BMC Complement. Altern. Med.* **2012**, *12*, 173, doi:10.1186/1472-6882-12-173.
36. Yılmaz, B.S.; Ozbek, H.; Citoğlu, G.S.; Uğraş, S.; Bayram, I.; Erdoğan, E. Analgesic and hepatotoxic effects of *Ononis spinosa* L. *Phytother. Res.* **2006**, *20*, 500–503.
37. Fiebich, B.L.; Grozdeva, M.; Hess, S.; Hüll, M.; Danesch, U.; Bodensieck, A.; Bauer, R. *Petasites hybridus* extracts *in vitro* inhibit COX-2 and $PGE_2$ release by direct interaction with the enzyme and by preventing p42/44 MAP kinase activation in rat primary microglial cells. *Planta Med.* **2005**, *71*, 12–19.
38. Bickel, D.; Röder, T.; Bestmann, H.J.; Brune, K. Identification and characterization of inhibitors of peptido-leukotriene-synthesis from *Petasites hybridus*. *Planta Med.* **1994**, *60*, 318–322.
39. Agosti, R.; Duke, R.K.; Chrubasik, J.E.; Chrubasik, S. Effectiveness of *Petasites hybridus* preparations in the prophylaxis of migraine: A systematic review. *Phytomedicine* **2006**, *13*, 743–746.
40. Lipton, R.B.; Göbel, H.; Einhäupl, K.M.; Wilks, K.; Mauskop, A. *Petasites hybridus* root (butterbur) is an effective preventive treatment for migraine. *Neurology* **2004**, *63*, 2240–2244.
41. Käufeler, R.; Polasek, W.; Brattström, A.; Koetter, U. Efficacy and safety of butterbur herbal extract Ze 339 in seasonal allergic rhinitis: Postmarketing surveillance study. *Adv. Ther.* **2006**, *23*, 373–384.
42. Grossmann, M.; Schmidramsl, H. An extract of *Petasites hybridus* is effective in the prophylaxis of migraine. *Int. J. Clin. Pharmacol. Ther.* **2000**, *38*, 430–435.
43. Thomet, O.A.R.; Simon, H.U. Petasins in the treatment of allergic diseases: Results of preclinical and clinical studies. *Int. Arch. Allergy Immunol.* **2002**, *129*, 108–112.
44. Guo, R.; Pittler, M.H.; Ernst, E. Herbal medicines for the treatment of allergic rhinitis: A systematic review. *Ann. Allergy Asthma Immunol.* **2007**, *99*, 483–495.
45. Lim, H.J.; Lee, H.S.; Ryu, J.H. Suppression of inducible nitric oxide synthase and cyclooxygenase-2 expression by tussilagone from Farfarae flos in BV-2 microglial cells. *Arch. Pharm. Res.* **2008**, *31*, 645–652.
46. Qin, Z.B.; Zhang, J.; Wu, X.D.; He, J.; Ding, L.F.; Peng, L.Y.; Li, X.Y.; Li, Y.; Deng, L.; Guo, Y.D.; *et al.* Sesquiterpenoids from *Tussilago farfara* and their inhibitory effects on nitric oxide production. *Planta Med.* **2014**, *80*, 703–709.
47. Hwangbo, C.; Lee, H.S.; Park, J.; Choe, J.; Lee, J.H. The anti-inflammatory effect of tussilagone, from *Tussilago farfara*, is mediated by the induction of heme oxygenase-1 in murine macrophages. *Int. Immunopharmacol.* **2009**, *9*, 1578–1584.
48. Keinänen, M.; Julkunen-Tiitto, R. High-performance liquid chromatographic determination of flavonoids in *Betula pendula* and *Betula pubescens* leaves. *J. Chromatogr. A* **1998**, *793*, 370–377.

49. Guardia, T.; Rotelli, A.E.; Juarez, A.O.; Pelzer, L.E. Anti-inflammatory properties of plant flavonoids. Effects of rutin, quercetin and hesperidin on adjuvant arthritis in rat. *Farmaco* **2001**, *56*, 683–687.
50. Germanò, M.P.; Cacciola, F.; Donato, P.; Dugo, P.; Certo, G.; D'Angelo, V.; Mondello, L.; Rapisarda, A. *Betula pendula* leaves: Polyphenolic characterization and potential innovative use in skin whitening products. *Fitoterapia* **2012**, *83*, 877–882.
51. Wacker, K.; Gründemann, C.; Kern, Y.; Bredow, L.; Huber, R.; Reinhard, T.; Schwartzkopff, J. Inhibition of corneal inflammation following keratoplasty by birch leaf extract. *Exp. Eye Res.* **2012**, *97*, 24–30.
52. Gründemann, C.; Gruber, C.W.; Hertrampf, A.; Zehl, M.; Kopp, B.; Huber, R. An aqueous birch leaf extract of *Betula pendula* inhibits the growth and cell division of inflammatory lymphocytes. *J. Ethnopharmacol.* **2011**, *136*, 444–451.
53. Wink, M. Evolutionary advantage and molecular modes of action of multi-component mixtures used in phytomedicine. *Curr. Drug Metab.* **2008**, *9*, 996–1009.
54. Mamani Matsuda, M.; Kauss, T.; Al Kharrat, A.; Rambert, J.; Fawaz, F.; Thiolat, D.; Moynet, D.; Coves, S.; Malvy, D.; Mossalayi, M.D. Therapeutic and preventive properties of quercetin in experimental arthritis correlate with decreased macrophage inflammatory mediators. *Biochem. Pharmacol.* **2006**, *72*, 1304–1310.
55. Huang, D.; Ou, B.; Prior, R.L. The chemistry behind antioxidant capacity assays. *J. Agric. Food Chem.* **2005**, *53*, 1841–1856.
56. Kukić, J.; Popović, V.; Petrović, S.; Mucaji, P.; Ćirić, A.; Stojković, D.; Soković, M. Antioxidant and antimicrobial activity of *Cynara cardunculus* extracts. *Food Chem.* **2008**, *107*, 861–868.
57. Katsube, T.; Tabata, H.; Ohta, Y.; Yamasaki, Y.; Anuurad, E.; Shiwaku, K.; Yamane, Y. Screening for antioxidant activity in edible plant products: Comparison of low-density lipoprotein oxidation assay, DPPH radical scavenging assay, and Folin-Ciocalteu assay. *J. Agric. Food Chem.* **2004**, *52*, 2391–2396.
58. Cai, Y.; Luo, Q.; Sun, M.; Corke, H. Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. *Life Sci.* **2004**, *74*, 2157–2184.
59. Wojdyło, A.; Oszmiański, J.; Czemerys, R. Antioxidant activity and phenolic compounds in 32 selected herbs. *Food Chem.* **2007**, *105*, 940–949.
60. Djeridane, A.; Hamdi, A.; Bensania, W.; Cheifa, K.; Lakhdari, I.; Yousfi, M. The *in vitro* evaluation of antioxidative activity, $\alpha$-glucosidase and $\alpha$-amylase enzyme inhibitory of natural phenolic extracts. *Diabetes Metab. Syndr.* **2013**, doi:10.1016/j.dsx.2013.10.007.
61. Hanekamp, W.; Lehr, M. Determination of arachidonic acid by on-line solid-phase extraction HPLC with UV detection for screening of cytosolic phospholipase $A_2\alpha$ inhibitors. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **2012**, *900*, 79–84.
62. Li, B.; Copp, L.; Castelhano, A.L.; Feng, R.; Stahl, M.; Yuan, Z.; Krantz, A. Inactivation of a cytosolic phospholipase $A_2$ by thiol-modifying reagents: cysteine residues as potential targets of phospholipase $A_2$. *Biochemistry* **1994**, *33*, 8594–8603.
63. Cleland, W.W. Dithiothreitol, a new protective reagent for SH groups. *Biochemistry* **1964**, *3*, 480–482.

64. Riendeau, D.; Guay, J.; Weech, P.K.; Laliberté, F.; Yergey, J.; Li, C.; Desmarais, S.; Perrier, H.; Liu, S.; Nicoll Griffith, D. Arachidonyl trifluoromethyl ketone, a potent inhibitor of 85-kDa phospholipase $A_2$, blocks production of arachidonate and 12-hydroxyeicosatetraenoic acid by calcium ionophore-challenged platelets. *J. Biol. Chem.* **1994**, *269*, 15619–15624.
65. Schmitt, M.; Lehr, M. HPLC assay with UV spectrometric detection for the evaluation of inhibitors of cytosolic phospholipase $A_2$. *J. Pharm. Biomed. Anal.* **2004**, *35*, 135–142.
66. Singleton, V.L.; Orthofer, R.; Lamuela-Raventós, R.M. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods Enzymol.* **1999**, *299*, 152–178.
67. Blois, M. Antioxidant determinations by the use of a stable free radical. *Nature* **1958**, *181*, 1199–1200.

*Sample Availability*: Samples of the methanol extracts or the $cPLA_2\alpha$ are not available.

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