The Andrographolide Content and Biological Activities of the Ethanolic Leaf Extract of *Andrographis paniculata* (Burm. f.) Ness

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Abstract
Andrographolide is a crystalline diterpene lactone present in Andrographis paniculata (AP) and considered as the most active and important secondary metabolite component of the said plant. Few reports are available regarding the biological activities of the AP plant extract. In this study andrographolide content of ethanolic extracts of different AP plant parts was determined using UPLC. COX-2 inhibitory activity and anti-oxidant property of the extract with highest andrographolide content were examined. COX-2 inhibition assay was done using fluorometric method and antioxidant activity was determined using DPPH method.

Highest content of andrographolide was observed in the ethanolic leaf extract. 150, 300, 600, 800 and 1000 µg/ml of ethanolic leaf extract of AP exhibited above 50% COX-2 inhibition and statistical analysis showed significant difference between their respective percent inhibition and the negative control. On the other hand, 100, 500, 750, 1000 and 2000 µg/ml of ethanolic leaf extract of AP exhibited 12.23, 27.08, 40.9, 46.85 and 51.67 % DPPH scavenging activity.

Keywords: *Andrographis paniculata and*rographolide, COX-2, DPPH, anti-inflammatory.

Introduction

For many years, natural products have been the backbone of traditional system of treatment of diseases worldwide and play a vital role in improving human health. They have been the drugs of choice due to their safety and efficacy despite the tough competition from synthetic compounds[26]. Newman and Cragg[17] cited in their review that for 30 years (January 1981-December 2010) from among the 1355 new compounds, 22 % (299) were semi-synthetic derived from natural products, 4 % (59) were natural products and 15 % (202) were biologicals or peptides. From 1940s to 2012, of the 175 small molecules used for cancer treatment, 131 or 74.8% were non-synthetic, with 85 or 48.6% actually are either natural products or directly derived there from.

Andrographis paniculata (Burm. f.) Nees is one of the most popular medicinal plants used traditionally for centuries in Asia, America and Africa for the treatment of an array of diseases[18]. It has anti-inflammatory[1,4,5], antimicrobial[22], anti-malarial[8,27], antithrombotic[25], antioxidant[16], hepatoprotective [15,29] and hypoglycemic activities [3,11]. Several biologically active compounds have been isolated and characterized from *AP* such as xanthones [8], flavonoids[6], arabinogalactan[7] and labdane type diterpenoids [6,18]. Among the secondary metabolites found in *AP* andrographolide, which is a colorless crystalline diterpene lactone, is considered to be the most active and important constituent [19,24].

Cyclooxygenase is a heme-dependent bifunctional enzyme that catalyzes the cyclization of arachidonic acid and reduction of Prostagladin G2 to Prostagladin H2. It exists in two forms namely COX-1, which is a constitutive form and COX-2, an inducible form. COX-1 enzyme is necessary for the maintenance of gastric integrity and kidney function whereas COX-2 is involved in inflammation and pain. Non-selective COX inhibitor that inhibits both COX-1 and COX-2 exhibited adverse ulcerogenic effects while COX-2 selective inhibitors showed strong anti-inflammatory effect and a much lower gastrointestinal side effects [9,14,21]. COX-2 was highly expressed in various cancers and now being considered as protein target for cancer therapy. Literature showed that direct binding of certain compound with COX-2 is absolutely required for its inhibition to human colon adenocarcinoma HT-29 cells [30].

Environmental pollution and unhealthy lifestyles lead to the generation of high levels of free radicals and reactive oxygen species (ROS) which can damage the structures and modify the functions of biomolecules. Free radicals and ROS can increase oxidative stress in systemic level which is manifested in variety of health problems such as cancer, age-related diseases and cardiovascular diseases[13]. Anti-oxidants help the system to neutralize free radicals and ROS thus lowering the risk of developing the health problems. This study determined the andrographolide content of *AP* plant from the Philippines and its COX-2 inhibitory activity and anti-oxidant property.

Material and Methods

Collection and Preparation of *AP:* The plant was identified by the Museum of Natural History as *Andrographis paniculata* (Burm. f.) Nees. Fully matured *AP* was collected from residential gardens in Batangas, Philippines. The clean leaves, stems, roots and fruits were air dried and further oven dried at 40 ºC. The dried samples were ground and sieved.

Chemicals and Standards: Andrographolide standard and 1,1-diphenyl-2-picrylhydrazyl (DPPH) purchased from Sigma Aldrich and HPLC grade methanol from Scharlau and analytical grade methanol and ethanol from JTBaker were used.

Determination of the Amount of Andrographolide by UPLC: The andrographolide content of the different plant parts of *AP* namely: leaves, roots, stems and fruits was determined to select the plant part to be utilized in the bio-assays. Each sample was prepared separately for UPLC analysis using the same extraction protocol. The 80-100 mesh of ground dried sample (1.00 ± 0.01 g) was extracted three times with 20mL of ethanol at room temperature (23 ◦C) in ultrasonic water-bath (at 40 ◦C for 45 min) and centrifuged (4500 rpm, 15 min). The centrifugates were combined and an aliquot was taken from each sample for UPLC analysis[25].

UPLC analysis was performed using the modified method of Kurzawa. UPLC Waters Acquity H with photo diode array (PDA) detector and reverse phase column (Acquity UPLC BEH C-18, 50 x 2.1 mm, 1.7 µm particle size) were used. Separation of sample components was done using a mixture of methanol-acetic acid 0.01 M (7:3, v/v) as mobile phase at a total flow rate of 0.3 mL/min in isocratic conditions and the sample injection volume of 1 µl. The absorption was measured at 230 nm. The analysis was done in triplicate and calibration curve was prepared using standard solutions of concentration range between 50 and 1000 µg mL^{-1}. The area of each peak was plotted as the function of concentration[12].

Cyclooxygenase-2 (COX-2) inhibition assay: The COX-2 inhibition assay was done using Fluorometric method. Samples were submitted to the Terrestrial Natural Products Laboratory, Center for Drug Discovery and Development, Institute of Chemistry, University of the Philippines Diliman for analysis.

To a 150 µL of 100 mM Tris, the following were added in order: 10 µl of 4,500 µg mL^{-1} of ethanolic leaf extract of *AP* in DMSO (to make a final well concentration of 150 µg mL^{-1}), 10 uL of 1,000 µM Hemin and 10 µL of 250 U/mL COX-2 enzyme. The positive control used was 10 µl of 2,862 µg mL^{-1} Indomethacin in 100% DMSO (final well concentration) and the negative control was 10 µL 100% DMSO. The mixture was incubated at 25 ºC for 15 minutes. After incubation, 10 µL of 200 uM amplex red was added to the mixture. Ten µL of 2,000µM of arachidonic acid was then added and the reaction was monitored for 2 minutes using the fluorescence mode of the Varioskan Flash (Thermo Scientific) with the excitation and emission wavelength set at 535 and 590 nm respectively.

The % inhibitory activity of the samples and the positive control were determined based on the average slope of each replicate using the following formula:

$$\% \text{ Inhibitory Activity} = \frac{\text{Slope}_{\text{uninhibited}} - \text{Slope}_{\text{inhibited}}}{\text{Slope}_{\text{uninhibited}}} \times 100$$

where Slope $_{\text{uninhibited}}$ is the slope of the line from the fluorescence intensity vs. time plot of the negative control group and the Slope $_{\text{inhibited}}$ is the slope of the line from the fluorescence intensity vs. time plot of the samples/positive control[10,23]. The method was also done for ethanolic leaf extract of *AP* at different effective well concentrations: 300, 600, 800 and 1,000 µg mL^{-1}.

Antioxidant Activity using DPPH assay: The free radical scavenging activity of different concentrations of ethanolic leaf extract of *AP* was determined *in-vitro* by 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay[20]. An aliquot of 2.5 ml of different concentration of sample solution in methanol was mixed with 2.5 ml of DPPH (0.16 mM in methanol). The control was prepared by adding 2.5 ml of DPPH in methanol with 2.5 ml of methanol. The reaction mixtures were mixed and incubated in the dark at room temperature for 30min. The absorbance was then read at 517nm using UV-vis Spectrophotometer (Shimadzu). The DPPH free radical scavenging activity (%) was calculated by using the formula:

(%) DPPH scavenging activity =
[(absorbance of control–absorbance of sample
---×100
(absorbance of control)]

Results and Discussion

Andrographolide content of different plant parts: The UPLC chromatograms of andrographolide standards showed two peaks. The first peak with a retention time of 0.424-0.426 minutes is for the solvent used to dissolve the standard which was ethanol while the second peak with retention time range of 0.610-0.613 minutes was for andrographolide. The UPLC chromatograms of ethanolic leaf, stem and fruit extracts of *AP* (Figure 1) showed that they had similar constituents. Their UPLC chromatograms showed three major components and one minor component.

The first peak represents the solvent which was ethanol while the second peak which was the major component of the three ethanolic extracts was identified as andrographolide. The third and fourth peaks were not identified due to lack of standards. This finding is parallel to that of Kurzawa et al[12] who also showed that the major component of *AP* ethanolic extract was andrographolide. Using C18 and a mobile phase of a mixture of methanol-acetic acid 0.01M (7:3v/v), they identified three diterpenes in *AP* ethanolic extract namely: andrographolide, deoxyandrographolide and neoandrographolide.

The ethanolic root extract of *AP* had more diverse components (Figure 2) as compared with leaf, stem and fruit ethanolic extracts. Okhuarobo and coworkers[18] reported that the *AP* root contains more constituents than the other parts of the plant. Similarly, Dua et al[8] also reported that the roots contain four xanthones namely 1,8-dihydroxy-3,7-dimethoxy-xanthone, 4,8-dihydroxy-2,7- dimethoxy-xanthone, 1,2-dihydroxy-6,8-dimethoxyxanthone and 3,7,8-trimethoxy-1-hydroxy-xanthone.

Xanthone skeleton usually gave strong UV absorptions at 310, 246 and 210 nm due to the extended chromophore and a substituted benzene ring[28]. The UV-absorption maxima (λ max) of xanthones are 245 and 323 nm. Based on the comparison of the chromatogram of the *AP* ethanolic root extract with that of the andrographolide standard, there was no peak with retention time range of 0.610-0.613 minutes. It is inferred that andrographolide is not present in the ethanolic root extract or may be present in a very low amount.

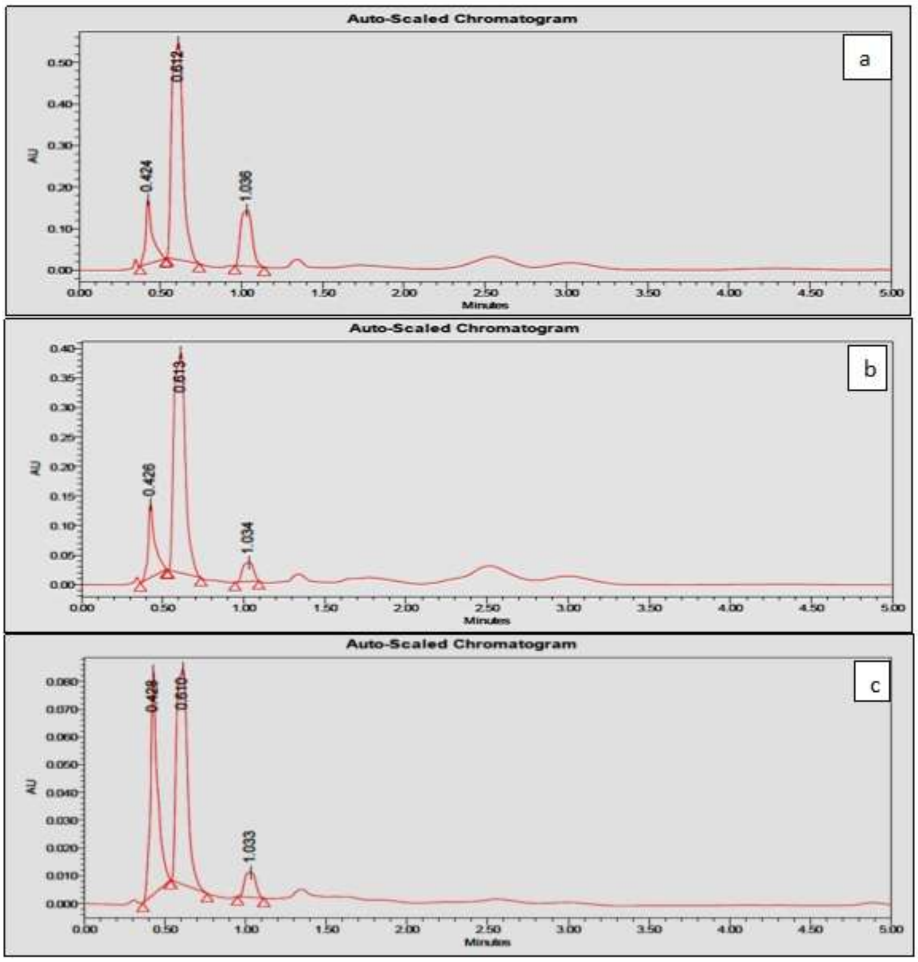

Figure 1: UPLC Chromatograms of ethanolic (a) leaf (b) stem (c) fruit extracts of *A.paniculata*

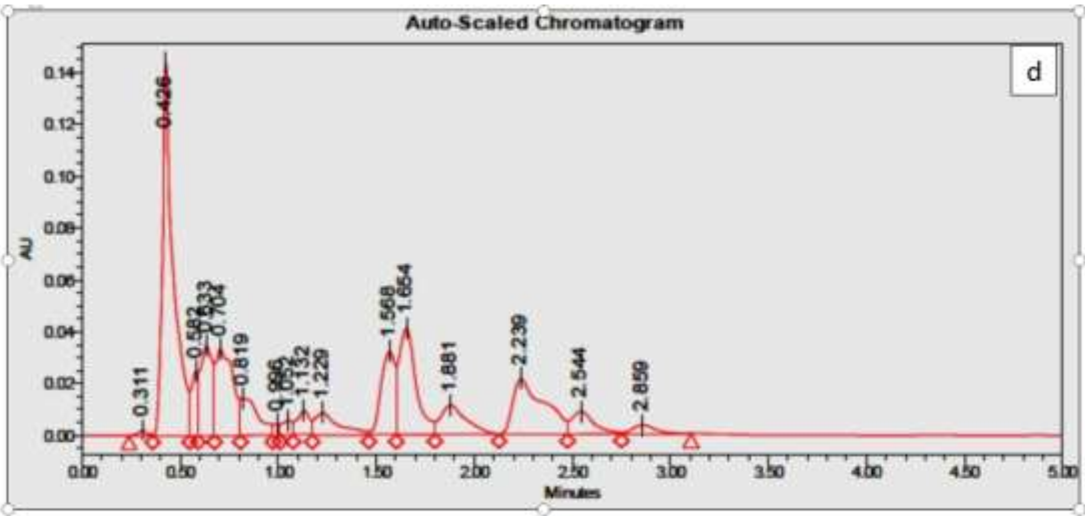

Figure 2: UPLC Chromatogram of ethanolic root extracts of *A.paniculata*

The andrographolide content of the different plant parts of *A. paniculata* in table 1 showed that the leaves had the highest amount with 22.09 ± 0.20 mg/g followed by the stem and the fruits had the lowest. Comparison of the levels of andrographolide among the three plant parts showed a calculated f-value of 12,240.209 and p value of 8.45×10^{-33} which indicates significant difference on the andrographolide content among the three plant parts.

Table 1
Andrographolide Content of Different Plant Parts of *A. paniculate*

Plant part	Andrographolide Content (mg/g) [a]
Leaves	22.09 ± 0.20*
Stem	16.38 ± 0.37 *
Fruits	3.98 ± 0.06*
Roots	Not Detected

Mean ± Standard deviation (n=6)
* indicating with significantly different (p< 0.05) with other plant part

The obtained andrographolide content of ethanolic leaf extract of *AP* samples from Batangas, Philippines was lower than that obtained by Kurzawa et al[12] using the ethanolic leaf extract of *AP* from pharmaceutical companies in Poland, which was reported to have 37.29 mg/g. The obtained andrographolide in the current study was found comparable to that obtained by Akowuah and coworkers[2] in the leaves of the *AP* cultivated in different locations of Malaysia which ranged from 21.50 to 29.50 mg/g. Differences in the andrographolide content may be attributed to genetic variation, geographic location and growing conditions of the plant sources.

COX-2 inhibitory activity: Different concentrations of the ethanolic leaf extract of *AP* were screened for possible COX-2 inhibitory activity. As shown in figure 3, all concentrations tested, 150, 300, 600, 800 and 1000 µg/ml exhibited above 50 % inhibition. Comparison of their respective percent inhibition with the negative control yielded the following p values range of 0.000-0.004 indicating ethanolic leaf extract of *AP* exhibited active inhibitory activity against COX-2.

In addition, the inhibitory activity of ethanolic leaf extract of *AP* was higher than that of indomethacin since it exhibited 71.15 % COX-2 inhibition at 150 µg/ml. The IC 50 of indomethacin was in the range of 364-1227 µg/ml and its concentration that yielded 89.54 % COX-2 inhibition was 2,862 µg/ml.

These findings may explain some reported pharmacological properties of *AP* leaves such as anti-inflammatory[1,4,5] and anti-cancer[18,30]. The ability of *AP* leaf extract to alleviate pro-inflammatory, inflammatory and allergic mediators and its anti-cancer and anti-tumor activities can be attributed to its COX-2 inhibitory property.

Antioxidant activity in terms of % DPPH scavenging: DPPH is a stable radical in solution and appears with a purple color absorbing at 515 nm in methanol. In the presence of scavenger molecule i.e. antioxidant, DPPH accepted a hydrogen (H) atom which resulted to the reduction of DPPH to DPPH2, manifested by conversion of purple color to yellow with concomitant decrease in absorbance at 515 nm[13]. As shown in figure 4, all concentrations tested exhibiting high % DPPH scavenging activity and this activity increases with ethanolic leaf extract concentration.

In addition, the observed % DPPH scavenging activity in this study was higher than that obtained by Sangeetha et al[20] in hexane, ethyl acetate, chloroform and methanol *AP* leaf extracts with 10.09 ± 0.16, 35.08 ± 0.24, 24.26 ± 0.18 and 49.04 ± 0.12 % respectively at 100,000 µg/ml.

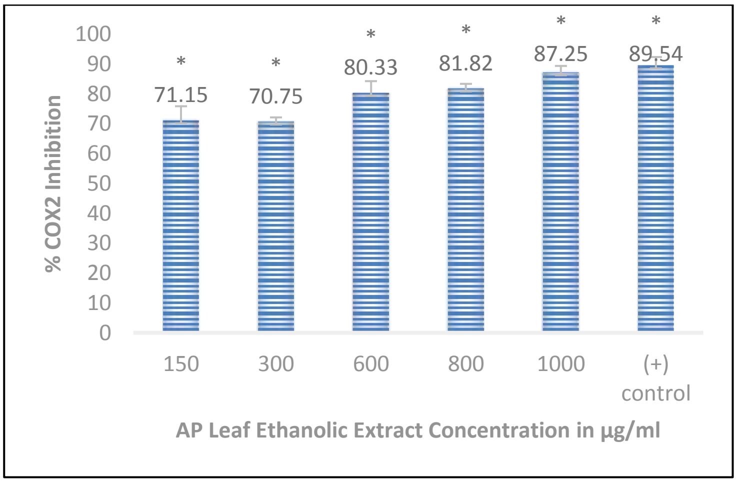

Figure 3: COX-2 inhibitory activities of different concentrations of ethanolic leaf extract of *A. paniculata*. Data were based on average values (n=4) ± SD, with * indicating significant difference with negative control at p<0.05. Sample is considered "active" if the COX-2 percent inhibition is greater than or equal to 50% and if the COX-2 activity has a significant mean difference compared to negative control. Positive control used was Indomethacin at 2,862 µg/ml.

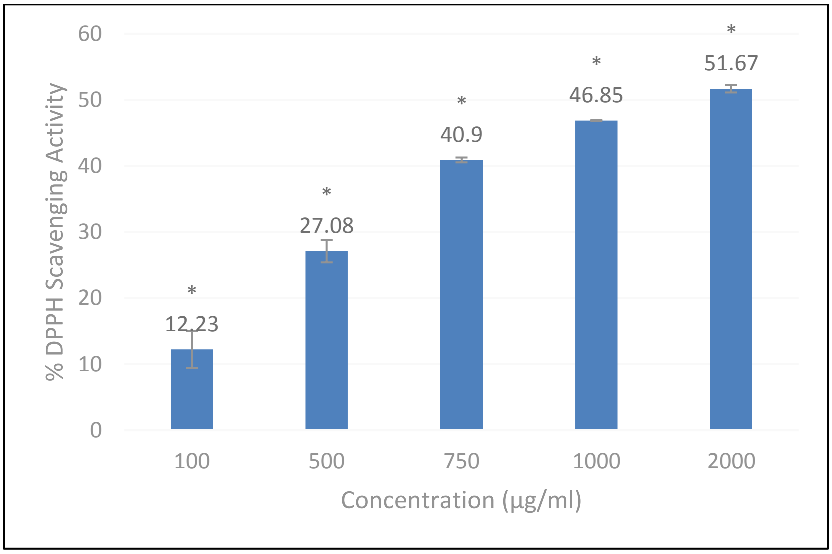

Figure 4: Antioxidant activities of different concentrations of ethanolic leaf extract of *A. paniculata* . Data were based on average values (n=3) ± SD, with * indicating significant difference with negative control at $p<0.05$

The difference in the levels of % DPPH scavenging activity may be attributed to the solvent used in extraction and to the genetic variation, geographic location and growing conditions of the plant sources.

Conclusion

The andrographolide content of *AP* differs significantly among its plant parts. The leaves contain the highest amount of andrographolide with 22.09 ± 0.20 mg/g. The ethanolic leaf extract of *AP* exhibits significant COX-2 inhibitory activity and anti-oxidant activity. These findings could explain some of the reported pharmacological properties of *AP* leaves.

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