An *in vitro* study of anti-inflammatory activity of standardised *Andrographis paniculata* extracts and pure andrographolide

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**Abstract**

**Background:** The anti-inflammatory activity of *Andrographis paniculata* (Acanthaceae), a traditional medicine widely used in Asia, is commonly attributed to andrographolide, its main secondary metabolite. Commercial *A. paniculata* extracts are standardised to andrographolide content. We undertook the present study to investigate 1) how selective enrichment of andrographolide in commercial *A. paniculata* extracts affects the variability of non-standardised phytochemical components and 2) if variability in the non-standardised components of the extract affects the pharmacological activity of andrographolide itself.

**Methods:** We characterized 12 commercial, standardised (≥30% andrographolide) batches of *A. paniculata* extracts from India by HPLC profiling. We determined the antioxidant capacity of the extracts using 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging, oxygen radical antioxidant capacity (ORAC) and a Folin-Ciocalteu (FC) antioxidant assays. Their anti-inflammatory activity was assessed by assaying their inhibitory effect on the release of tumor necrosis factor alpha (TNF-α) in the human monocytic cell line THP-1.

**Results:** The andrographolide content in the samples was close to the claimed value (32.2 ± 2.1%, range 27.5 to 35.9%). Twenty-one non-standardised constituents exhibited more than 2-fold variation in HPLC peak intensities in the tested batches. The chlorogenic acid content of the batches varied more than 30-fold. The DPPH free radical scavenging activity varied ~3-fold, the ORAC and FC antioxidant capacity varied ~1.5 fold among batches. In contrast, the TNF-α inhibitory activity of the extracts exhibited little variation and comparison with pure andrographolide indicated that it was mostly due to their andrographolide content.

**Conclusions:** Standardised *A. paniculata* extracts contained the claimed amount of andrographolide but exhibited considerable phytochemical background variation. DPPH radical scavenging activity of the extracts was mostly due to the flavonoid/phenylcarboxylic acid compounds in the extracts. The inhibitory effect of andrographolide on the release of TNF-α was little affected by the quantitative variation of the non-standardised constituents.

**Keywords:** Andrographolide, *Andrographis paniculata*, Anti-inflammatory, Antioxidant, TNF-α, Phytochemistry

**Background**

Chronic inflammation is thought to be a contributing factor to many prevalent ageing-related diseases, such as acute and chronic neurodegenerative diseases, degenerative musculoskeletal diseases, cardiovascular diseases, diabetes, and cancer [1-5]. Other common chronic inflammatory conditions include asthma, rheumatoid arthritis, and inflammatory bowel disease. To date, pharmacotherapy of inflammatory conditions is based mainly on the use of non-steroidal anti-inflammatory drugs (NSAIDs), steroids and more recently tumor necrosis factor alpha (TNF-α) inhibitors [6-8]. However, the prolonged use of NSAIDs can cause serious gastrointestinal toxicity [9]. Some NSAIDs have also been linked to increased blood pressure, greatly increased risk of congestive heart failure and occurrence of thrombosis [10]. These findings illustrate the need to develop novel and safe anti-inflammatory medicines [11].
Andrographis paniculata (Acanthaceae), which is endogenous to South India and South East Asia, is used as an herbal medicine in both traditional Indian and Chinese medicine (where it is known as kalmegh and chuanxilin, respectively) as well as in Malaysia and Thailand [12,13]. A. paniculata extracts exhibit anti-inflammatory activity [13] that is commonly attributed to the ent-labdane diterpenoid andrographolide, its characteristic and main secondary metabolite [14-30]. Andrographolide appears to be rapidly absorbed [31] and found to be non-toxic even at very high doses in animals [32] and is well tolerated by humans with no serious adverse effects at doses in the range of 1 to 2 mg/kg/day [33,34]. Intriguingly, andrographolide has been reported to exhibit gastro-protective and ulcer preventive effects, which combined with its well-documented anti-inflammatory effects could make it a safe alternative to traditional NSAIDs [35]. A proprietary A. paniculata extract (HMPL-004, Hutchison MediPharma) is under development for the treatment of inflammatory bowel disease [36,37] and is currently being tested in a global phase III clinical trial (http://clinicaltrials.gov/show/NCT01805791).

Commercial A. paniculata tablets standardised to requisite concentrations of andrographolide (5% or 30% w/w) are used in clinical studies with an assumption of consistency [38,39]. However with reported innate phytochemical variation influenced by phytogeographical and spatiotemporal factors [40-44], it is not known how selective enrichment (standardization) of andrographolide in commercial preparations affects the variability of non-standardised phytochemical components. It is also not known if variations in the non-standardised components affect the anti-inflammatory activity of the extracts. We undertook the present study to investigate 1) how selective enrichment of andrographolide in commercial A. paniculata extracts affects the variability of non-standardised phytochemical components and 2) if variability in the non-standardised components affects the pharmacological activity of the extracts. To this end, we profiled the phytochemical composition and antioxidant capacity of standardised A. paniculata extracts and compared the activity of the extracts and purified andrographolide in an assay relevant to their anti-inflammatory activity.

Anti-inflammatory activity of andrographolide has been studied using a number of in vivo and in vitro experimental paradigms including human whole genome DNA microarrays [20]. The most commonly implicated molecular mechanism underpinning the anti-inflammatory and immunomodulatory effects of andrographolide is inhibition of the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) signalling (specifically p38 MAPK/ERK1/2) pathway and downstream transcription factors such as nuclear factor kappa B (NF-kB) and nuclear factor of activated T cells (NFAT) [24,45-50]. An exemplary experimental in vitro model where this mechanism has been implicated is the inhibition by andrographolide of the release of TNF-α from LPS stimulated macrophages [22,51-54]. Therefore, we chose TNF-α release from LPS stimulated monocytc leukaemia cells (THP-1) as a model to study anti-inflammatory activity of the extracts and purified andrographolide.

Methods

Extract provenance and preparation

Twelve commercial batch samples of A. paniculata extracts (extract ratio 14:1) standardised to ≥30% andrographolide were kindly provided to us by LIPA Pharmaceuticals Ltd (NSW, Australia). The extracts conform to the TGA guidelines for incorporation in herbal medicines manufactured in Australia. The whole plant starting material for each batch was sourced (during 2004 – 2008) and the extracts manufactured in India. A systematic botanist authenticated each batch's starting material and the manufacturer provided traceability documents for each extract. All the samples were re-analysed by high-performance liquid chromatography (HPLC) with photodiode array detection (PDA) for andrographolide content to reconfirm the manufacturer's certificate of analysis.

Phytochemical analysis

We used HPLC to profile the phytochemical composition of the A. paniculata extracts. Andrographolide (14.3 mg in 10 ml) and extract samples (125 mg in 50 ml) were dissolved by sonication in methanol. HPLC analysis of andrographolide and the extract samples was performed using a Varian Inc. (USA) HPLC system equipped with ProStar 335 photodiode array detector (PDA) and 1200 L quadrupole tandem mass spectrometry (MS/MS) detector. An Alltech Alltima (Alltech Australia) reverse phase C18 column (46 × 150 mm I.D., 5 µm) with a Phenomenex (California, USA) Security C18 guard column (20 mm x 4 mm, 5 µm) were used in these experiments.

We generated HPLC-PDA and HPLC-MS/MS profiles using a 5 µl injection of extract samples. The mobile phase consisted of 0.1% (v/v) aqueous formic acid (mobile phase A) and 0.1% (v/v) formic acid in acetonitrile (mobile phase B). The mobile phase gradient was 10% B for 10 min with a linear increase; to 50% B at 63 min, 70% B at 72 min and then 100% B (wash) for 8 min before equilibrating at the starting composition for 5 min. Mobile phase flow rate was maintained at 1 ml/min. The post column flow was split to send 80% to the PDA (200–500 nm) and 20% to the MS.

The MS conditions were adapted from the work of Dong et al. [55]. Ionization was achieved in positive electrospray ionization mode, scanning between 70–700 m/z.
with the needle voltage 5000 V at 13 μA; nebulization gas (nitrogen) temperature of 350°C at 20 psi; shield voltage 175 V; capillary voltage 53 V and the detector voltage was 1600 V.

We quantified the andrographolide (0.14 - 1.4 mg/ml) content of the extracts at a detection wavelength of 240 nm and the chlorogenic acid (6.6 – 132.4 μg/ml) content at 330 nm, using five-point linear calibration curves. We generated chromatograms at 227 nm for the detection of diterpenes and at 261 nm and 330 nm for the detection of flavonoids and phenyl carboxylic acids, respectively.

While the PDA was used to quantify andrographolide and chlorogenic acid, the MS detector was used to confirm the identity of these peaks by comparing the MS/MS obtained for the sample and reference standard peaks. MS/MS data was also used to tentatively identify other diterpenes by comparison to published MS data. The UV spectra were used to assign tentatively some of the observed peaks as flavonoids, phenylcarboxylic acids or diterpenes as shown in Table 1.

We used the package ‘msProcess’ [56] (R Project) for Statistical Computing [57] to remove instrumental noise and baseline drift from the chromatograms as described in detail previously [58].

Antioxidant assays
We performed the DPPH (2,2-di(4-tert-octylphenyl)-1-picyrhydrazyl) radical scavenging capacity, oxygen radical absorbance capacity (ORAC) and the total phenol assay (Folin-Ciocalteu assay; FC) on the extracts as reported in detail previously [58,59].

Cell culture and tumor necrosis factor α (TNF-α) assay
We cultured human monocytic leukaemia cells (THP-1; American Type Culture Collection, Manassas, VA, USA) in RPMI (Roswell Park Memorial Institute) media containing 4.5 g/l D-Glucose and supplemented with 2 mM GlutaMax, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% foetal bovine serum at 37°C, 5% CO2 in 95% air. Cells (passage number between 10 and 25) were seeded at a density of 1 x 105 cells/well and incubated for 48 h in phorbol-12-myristate-13-acetate (PMA; 100 nM). Non-adherent cells were removed by washing with fresh medium. The remaining cells were preincubated with different concentrations of extracts or andrographolide for 1 h and stimulated by lipopolysaccharide (LPS; 50 ng/ml) and interferon gamma (50 units) for 24 h. Andrographolide and extracts were prepared in DMSO and added at a final concentration of 0.1% DMSO. We determined the concentration of TNF-α in the THP-1 culture supernatant by a commercial sandwich ELISA following the manufacturer’s instructions (PeproTech Inc., Rocky Hill, NJ, USA).

Potential cytotoxicity of the extracts and andrographolide was investigated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. No toxic effects of the extracts or andrographolide were observed at the tested concentrations (data not shown).

Statistical data analysis
All data is reported as mean ± standard deviation of the average of three replicates in experiments performed on three separate days. The TNF-α dose response data were fitted with a log (inhibitor) vs. normalized response with variable slope model using Prism 5 for Mac OSX (GraphPad Software, La Jolla, CA). IC50 values were calculated from the fitted curves. F-test was used to compare if the best-fit values differed between the data sets. Differences were considered statistically significant if p < 0.05.

Results and discussion
The phytochemical composition of 12 A.paniculata extracts was characterized by HPLC-PDA (Figure 1A). Consistent with the intended standardization, to contain 30% andrographolide, the content was on average close to this value (32.2 ± 2.1%, range 27.5 to 35.9%; n = 12; peak #12 in Figure 1B). Correlation analysis of the andrographolide concentration versus storage time revealed that there was no observable loss in andrographolide concentration due to storage of the dry extracts (r² = 0.03; not illustrated). Non-standardised constituents other than diterpenes (e. g. peaks 1–11), varied to a greater degree between the batches (Table 1). For example, the chlorogenic acid content exhibited >30 fold variation between batches (0.1 to 3.5 mg per g of extract; mean 1.4 ± 1.1 mg/g). The maximum variation observed between the chromatograms is illustrated by the red curve in Figure 1B.

The phytochemical analysis of the extracts revealed considerable variability in the peak intensities of the non-standardised constituents, some of which belonged to the flavonoid and phenylcarboxylic acid class. Flavonoids and phenolic acids are known to be good free radical scavengers and antioxidants [60]. Oxidative stress is believed to contribute to inflammatory tissue damage and play a role cytokine signalling [61]. We therefore wondered to what degree the observed phytochemical variation might be reflected in the antioxidant capacity of the extracts [62]. We used three non-cellular assays to measure the DPPH radical scavenging capacity, oxygen radical absorbance capacity (ORAC) and total phenol content (FC assay) [58]. The average DPPH radical scavenging capacity of the total extracts was 81.6 ± 30.9 μmol/g gallic acid equivalent and varied 3 fold. DPPH reactivity was correlated with the variation in chlorogenic acid content (r² = 0.8). Online-HPLC revealed that the DPPH
scavenging was mainly due to peaks 1–11 (flavonoids and phenylcarboxylic acids) while the diterpenes (peaks #12, 13, 20, 24 and 25) were virtually devoid of DPPH radical scavenging capacity under the conditions of our experiments (Figure 2). The average ORAC of the extracts was 1.05 ± 0.16 mmol/g gallic acid equivalent and varied 1.6 fold. The average antioxidant activity of the extracts in the FC assay was 0.40 ± 0.05 mmol/g gallic acid equivalent and varied 1.5 fold.

Next, we assayed the activity of the extracts and purified andrographolide on the inhibition of TNF-α release by LPS stimulated THP-1 cells. For this experiment, we obtained dose response curves of purified andrographolide and the extracts #3 and #9, the extracts with highest and lowest DPPH free radical scavenging activity. The results revealed that the dose response curves obtained upon application of pure andrographolide or the extracts were very similar (Figure 3), when normalized to

| Peak identity | Rt (min) | % Area | Fold variation | UV peaks (nm) | MS fragmentation (m/z) | Tentative assignment |
|---------------|----------|--------|----------------|--------------|------------------------|---------------------|
| Chlorogenic acid | 12.2 | 1.0 | 34.3 | 327, 218, 235 | Not determined (ND) | Phenyl carboxylic acid |
| Isoquercetin | 28.7 | 0.9 | 5.1 | 204, 255, 353 | ND | Flavonol glycoside |
| Peak 3 | 29.2 | 0.6 | 9.8 | 323, 219, 234 | ND | Phenyl carboxylic acid |
| Peak 4 | 29.7 | 3.5 | 3.8 | 325,219,235 | ND | Phenyl carboxylic acid |
| Peak 5 | 31.0 | 1.4 | 3.4 | 326, 219, 271 | ND | Phenyl carboxylic acid |
| Peak 6 | 32.2 | 4.1 | 6.1 | 324, 219, 234 | ND | Phenyl carboxylic acid |
| Peak 7 | 32.4 | 3.3 | 4.5 | 325, 220 | ND | Phenyl carboxylic acid |
| Peak 8 | 32.8 | 3.3 | 4.7 | 346, 224, 256 | ND | Phenyl carboxylic acid |
| Peak 9 | 33.7 | 2.1 | 4.5 | 204, 336, 266 | ND | Flavone |
| Peak 10 | 36.8 | 1.3 | 6.6 | 327, 219, 235 | ND | Phenyl carboxylic acid |
| Peak 11 | 37.7 | 0.8 | 4.4 | 327, 271, 223, 204 | ND | ND |
| Andrographolide | 38.4 | ND | ND | 227 | 351 [M+H]+, 333 [M-H2O]+, 315 [M-2H2O]+ | Diterpene |
| Andropanoside | 39.8 | 1.4 | 4.8 | 207 | 535 [M+K]+ | Diterpene |
| Peak 14 | 40.2 | 8.0 | 7.1 | 225 | ND | ND |
| Peak 15 | 40.7 | 4.3 | 3.7 | 202 | ND | ND |
| Apigenin | 42.2 | 2.6 | 6.7 | 211, 337, 267 | ND | Flavone |
| Wogonoside | 45.2 | 0.7 | 2.3 | 265, 212 | ND | O-methylated flavone glycoside |
| Peak 18 | 46.6 | 0.7 | 4.6 | ND | ND | ND |
| Peak 19 | 46.8 | 0.8 | 2.5 | ND | ND | ND |
| Neoandrographolide | 47.7 | 9.3 | 2.7 | 201 | 503 [M+Na]+, 519 [M+K]+, 319[M+H-Glu]+ | Diterpene |
| Peak 21 | 48.6 | 0.5 | 3.5 | ND | ND | ND |
| Peak 22 | 49.7 | 1.5 | 5.3 | ND | ND | ND |
| Peak 23 | 50.2 | 0.76 | 7.7 | ND | ND | ND |
| Deoxyandrographolide | 52.1 | 6.7 | 3.7 | 200 | 357 [M+Na]+, 317 [M+H2O]+, 299 [M+H-H2O]+ | Diterpene |
| Dehydroandrographolide | 52.6 | 35.1 | 2.1 | 200, 249 | 355 [M+Na]+, 315 [M+H2O]+, 297 [M+H-H2O]+ | Diterpene |
| Peak 26 | 53.6 | 0.8 | 2.8 | 229 | ND | ND |
| Peak 27 | 54.3 | 2.0 | 6.1 | 229 | ND | ND |
| Peak 28 | 54.6 | 2.8 | 3.0 | 200, 263 | ND | ND |

1UV peaks are listed in order of intensity.
2Fold variation = (Max. peak area) / (Min. peak area).
3Percent (%) area = average peak area from the 12 batches / total combined area × 100. Andrographolide was excluded from the % area calculations.
4Assignments based on UV spectrum, MS fragmentation and/or comparison to reference standard RT.
5MS fragments are listed in order of intensity.
The half maximal inhibitory concentration (IC₅₀) of pure andrographolide was 21.9 μM (n = 3; 95% confidence interval: 18.1 - 26.5 μM) compared to 16.4 μM (n = 3; 95% confidence interval: 13.8 - 18.8 μM) and 18.7 μM (n = 3; 95% confidence interval: 14.9 - 23.4 μM) for extracts #3 and #9, respectively. Thus, the phytochemical background variation of the extracts appeared not to influence significantly the activity of andrographolide in this in vitro assay.

We characterized the phytochemical composition of 12 batches of commercial *A. paniculata* extracts standardised for andrographolide content (30% w/w). We confirmed that the extracts contained the specified amount of andrographolide but observed substantial variation in the non-standardised components of the extracts. Chlorogenic acid exhibited maximal (~30 fold) variation. This acid is one of the most abundant phenolic compounds in the human diet and is present in significant amounts in coffee [63]. Various pharmacological effects of chlorogenic acid have been described and recent interest has focused on its effects on glucose and lipid metabolism [63]. Hypoglycaemic and hypocholesterolemic effects of water and ethanol *A. paniculata* extracts have been reported but the role of chlorogenic acid and its other phytochemical constituents and the molecular mechanisms underpinning these effects remain to be established [64]. Patients with diabetes taking *A. paniculata* extracts may therefore need extra monitoring and dietary counselling upon commencing or changing *A. paniculata* extract containing medications due to batch-to-batch variability of chlorogenic acid and its potential effects on blood glucose levels.

Our results revealed that of the commonly used in vitro antioxidant assays the DPPH assay was a more...
There are many anti-inflammatory compounds reported in *A. paniculata* [52,65,66] but andrographolide is the most abundant [55]. In this study the most dissimilar extracts in terms of DPPH activity and chemical profile (#3 and #9) were compared to pure andrographolide to assess their inhibition of TNFα release from LPS stimulated macrophages. Our comparison between the purified andrographolide and extracts containing parallel amounts of andrographolide in this assay found very similar dose response curves. Thus, at this level of analysis, there was no evidence for antagonistic, additive or synergistic effects between andrographolide and other phytochemical constituents of the extracts. The extract activity was almost entirely accounted for by the andrographolide content, thus the contribution of the other anti-inflammatory compounds present was minor, likely due to their lower abundance.

The IC$_{50}$ of andrographolide (21.9 μM) was comparable to values obtained in a similar assay using mouse peritoneal macrophages [51]. A number of additional *in vitro* studies using various experimental paradigms have all reported effective concentrations of pure andrographolide in the range of 7 to 35 μM [20,24,45,47,48,66-68]. The concentration reported in ours and other *in vitro* studies are nominal concentrations and the “actual” concentration at the site of interaction between andrographolide and its potential (extra- and/or intracellular) target proteins (receptor(s) or enzymes) has not been determined. Nonetheless, it is noteworthy that the IC$_{50}$ of andrographolide in our *in vitro* assay was ~50 times higher than the maximal plasma concentration (0.5 μM) achieved following oral administration of 50 mg andrographolide in healthy human volunteers [69], although significantly higher steady state blood concentrations (1.9 μM) have been reported in humans taking ~1 mg andrographolide per kg body weight per day [31]. In a prospective clinical study for the relief of rheumatoid arthritis symptoms, Burgos and colleagues administered 3 times per day 100 mg of *A. paniculata* extract standardised to 30% andrographolide [38]. Although these authors observed some positive effects, the *in vitro* data suggest that the administered dose might have been at the low end of the effective dose range and future clinical studies should consider testing higher doses. It will be interesting to investigate whether or not the absorption, distribution, metabolism and excretion of andrographolide alone or andrographolide administered as *A. paniculata* extract (with variable phytochemical background) differ or not.

**Conclusion**

Supported by a considerable body of published evidence, standardisation of *A. paniculata* extracts for andrographolide content is based on the notion that this

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**Figure 3** Dose response curves for batches #9 (blue, squares), #3 (red, circles) and andrographolide (green, triangles). IC$_{50}$ of pure andrographolide: 21.9 μM (n=3; 95% confidence interval: 18.1 - 26.5 μM), extract #3: 16.4 μM (n=3; 95% confidence interval: 13.8 - 18.8 μM), extract #9: 18.7 μM (n=3; 95% confidence interval: 14.9 - 23.4 μM). The concentrations have been normalized to concentration of andrographolide in the batches. The data were fitted with a log (inhibitor) vs. normalized response with variable slope model. The IC$_{50}$ was calculated from the fitted curve. F-test indicated that all data could be equally fitted with a single curve with IC$_{50}$ = 18.5 μM (p = 0.51; 95% confidence interval: 16.7 to 20.7 μM).
compound accounts for the pharmacological effects of the complex extracts. To the best of our knowledge, however, our data represent the only quantitative direct comparison of the efficacy of pure andrographolide and A. paniculata extracts. Thus, our results support the development of andrographolide or andrographolide-derived compounds as anti-inflammatory drugs. Interestingly, A. paniculata related drug development efforts presently include both herbal medicine based approaches in the form of standardised complex extracts as well as orthodox, synthetic drug based approaches [40,70-79]. We believe that well characterized standardised A. paniculata extracts present an excellent opportunity to further investigate the advantages and disadvantages of the herbal vs. synthetic approach in the treatment of inflammatory conditions.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
ML carried out the cell culture experiments and phytochemical analysis, participated in data analysis and manuscript writing. CK participated in the phytochemical and data analysis. GM, SG and NJS conceived the study, and collaborated in its design and coordination. NJS wrote the initial and final draft of the manuscript. All authors read and approved the final manuscript.

Acknowledgements
We thank Mr. Dusko Pejnovic, Chief Executive Officer of LIPA Pharmaceuticals Ltd. for his support, and LIPA Pharmaceuticals for providing samples of A. paniculata extracts. This study was partially supported by a Research Partnership Grant from the University of Western Sydney and LIPA Pharmaceuticals (NJS) and a UWS RGS grant (NJS and GM). We thank Mr. Dusko Pejnovic, Chief Executive Officer of LIPA Pharmaceuticals (NJS) and a UWS RGS grant (NJS and GM). We also thank Mr. Dusko Pejnovic, Chief Executive Officer of LIPA Pharmaceuticals for providing samples of A. paniculata extracts. This study was partially supported by a Research Partnership Grant from the University of Western Sydney and LIPA Pharmaceuticals (NJS) and a UWS RGS grant (NJS and GM).

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Received: 7 April 2014 Accepted: 15 January 2015
Published online: 07 February 2015

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