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Original research article

Inflammatory cytokines and metabolite changes after high dose of *Andrographis paniculata* extract: A preliminary study in mild COVID-19 case patients

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ABSTRACT

The Coronavirus disease which began in 2019 (COVID-19) was declared a global pandemic. It can increase inflammatory cytokines leading to organ failure. Efforts are being made to find drugs to treat or alleviate the severity. *Andrographis paniculata* (AP) is an herbal remedy for the respiratory system (cool tonic) in Thai traditional medicine theory. AP extracts have been reported to show potent inhibitory effects against SARS-CoV-2. The study aimed to investigate inflammatory cytokine changes and metabolomics profiling after high-dose AP extract had been given to patients with mild cases of COVID-19. Five patients received a high dose of AP extract (andrographolide 180 mg/day) for five consecutive days. The results showed that inflammatory cytokines related to COVID-19 infection tended to decrease. The metabolite profile showed a decrease of inflammatory metabolites and nucleotides. Moreover, the severity of the clinical symptoms was decreased within three days, especially cough. Side effects were not found by comparing the blood chemical analysis to pre-treatment controls. Therefore, a high dose of AP could be safely used in mild case COVID-19 patients. However, the absence of a placebo control meant that levels of pro-inflammatory cytokines and metabolite biomarkers related to clinical symptoms could not be determined. Therefore, further studies with comparative groups are needed and the number of patients studied should be increased.

Keywords: COVID-19, SARS-CoV-2, cytokines, metabolomics, herbal medicine, traditional medicine

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1. Introduction

The long-lasting Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2 or COVID-19) outbreak has become a pandemic since December 2019. It is spreading all over the world, resulting in rapidly growing numbers of patients with high mortality. SARS-CoV-2 is a new viral pathogen of humans, causing common cold-like illness and upper respiratory tract infection. Most COVID-19 infections are asymptomatic and severe symptoms are found only in 20% of patients, depending on the immune response.[1,2] Cytokine-release syndrome (CRS), informally called "cytokine storm", often occurs in SARS-CoV-2 infection because of massive release of inflammatory cytokines due to multiple organ failure.[3] Abnormal levels of cytokines and chemokines (IL-1, IL-2, IL-4, IL-6, IL-7, IL-10, IL-12, IL- 13, IL-17, M-CSF, G-CSF, GM-CSF, IP-10, IFN-γ, MCP-1, MIP-1α and TNF-α) are found in COVID-19 patients. Furthermore, vascular endothelial growth factors (VEGF), especially IL-12, IL-7, IL-10, G-CSF, IP-10, MIP-1α, MCP-1, and TNF-α are also signifi-cantly increased.[4-8] This finding of CRS induced by SARS-CoV-2 infection was helpful in that it has guided clinicians to focus on immuno-modulatory therapies for this infection.

Metabolomics is the study of metabolism in living organism biofluid including the human body and immune response through metabolites such as in the blood. Metabolites are small molecular products of human body transformation which rely on biochemical activities or phenotypes.[9] High-resolution mass spectrometry (MS) is used to provide metabolite profiles. Interest-ingly, metabolomics has become a potential tool to study COVID-19 infection regarding the disease progression, clinical prognosis and treatment. At present, there is no specific antiviral agent against SARS-CoV-2.[10]

According to Thai traditional medicine, COVID-19 patients encounter mainly 'fire' composition for which 'cold' type herbs, such as *Andrographis paniculata* (AP),[11] should be used to combat the illness. AP is an herbal remedy for the respiratory system in the National List of Essential Medicines. 2000 mg/day of AP (andrographolide 60 mg/day) is commonly used in common cold, sore throat, and diarrhea.[1,12] It has already been reported to show various pharmacological activities, such as antiviral,[13-17] immunostimulatory,[18,19] anti-inflammatory,[20-22] antidiarrheal,[23] antioxidant,[24,25] anticancer[18,26] and antihyperglycemic effects.[27] Andrographolide is the major bioactive constituent of AP.[28] Several studies reported that AP extract and andrographolide have a potent inhibitory effect against SAR-CoV-2.[29-31] The essence in SARS-CoV-2 infection could be the depletion of antiviral defenses related to immune response and an elevated production of inflammatory cytokines.

A study in humans reported that a high dose of AP (360 mg/kg) in one day can reduce fever.[32] Since COVID-19 is a new pandemic disease, a high dose (3 times higher than the normal dose described in the National List of Essential Medicines) was given to the patients. This study has investigated the changes of cytokines, chemokines, growth factors, and metabolite profiles after receiving a high dose of AP extract (andrographolide constituent 180 mg/day for 5 consecutive days) in mild cases of COVID-19, in order to study the efficacy and safety of AP extract.

2. Materials and Methods

2.1 AP drug

AP extract capsules containing at least 20 mg andrographolide were produced and their quality controlled by the PICs/GMP certified Manufacturing Unit of Thai herbal products Co., Ltd. (Phytocare) (a subsidiary of the Government Pharmaceutical Organization). HPLC analysis was performed to identify the quantity and the quality of the active compounds, including andrographolide, neoandrographolide, 14-deoxy-11 12-didehydroandrographolide, and andrograpanin (Supplementary S1).

2.2 Participants and drug administration

This is a descriptive uncontrolled before-after study. The protocols of the study were approved by the Ethics Committee of the department of Thai traditional and alternative medicine of the Ministry of Health of Thailand (SIDCER 05-2563). The study was conducted at Samutprakarn Hospital. Six participants were recruited and informed with written consent in this study. The nasal swabs of all participants were positive, as confirmed by real-time polymerase chain reaction (RT-PCR) within 72 hours. The inclusion criteria were: age 18-60 years, mild symptoms without pneumonia, and no requirement for antiviral treatment. The exclusion criteria were as follows: allergic to AP or other herbs that contain andrographolides, underlying disease such as hepatic disease, renal failure requiring dialysis, receiving immunosuppressants, congestive heart failure, hypertension, diabetes, COPD, or tuberculosis. The Thai Clinical Trials Registry (TCTR) identification number is TCTR20210914001.

Three AP capsules (20 mg/capsule) were given 3 times a day for 5 days. The clinical symptoms were assessed by numeric rating scale (0-10) including cough (severity, frequency), phlegm, sore throat, running nose, muscle ache and pain, headache, and difficulty in breathing. Blood and nasopharyngeal swabs for RT-PCR were collected on days 1, 3, and 5. After 5 days of AP treatment, the participants received standard treatment until discharge from hospital. The blood samples were used for cytokines, growth factors and chemokines measurement, and metabolomics profile analysis.

2.3 Cytokines, growth factors, and chemokines measurement

3-ml plastic Vacutainer spray-coated K2EDTA tubes (BD) (Becton Dickinson [BD], Franklin Lakes, NJ) were used to collect the participants' blood. The blood samples were centrifuged at 13,200 rpm for 10 min at 4°C to separate out the plasma. EDTA plasma samples were analyzed by using the BioPlex Pro Human Cytokine 27-Plex panel and the Bio-Plex 200 system (Bio-Rad, Hercules, CA, USA) following the manufacturer's instructions. The level of cytokines (IL-2, IL-4, IL-1β, TNF-α, IL-6, IFN-γ, IL-12, IL-7, IL-17, IL-13, IL-10, IL-8, IL-9, IL-15, IL-1ra, IL-5), growth factors (GM-CSF, VEGF, G-CSF, FGF basic, PDGF-bb) and chemokines (IP-10, MIP-1α, MCP-1, Eotaxin, RANTES, MIP-1b) were calculated with the Bio-plex manager software (Bio-Rad Laboratories).

2.4 Metabolite identification

Plasma samples that had been kept at -80°C were thawed on ice. 100 µL of plasma was mixed with 50 µL internal standard caffeine [3-METHYL-13C, 99% and cholic acid (2,2,4,4-D4, 98%)]. Liquid extraction of the samples was done by treating them with 300 µL methanol. The samples were vortexed and centrifuged at 15,800 g for 15 minutes at 4°C. The supernatant was collected and transferred to a vial.

LC/MS Q-TOF (Synapt G2-Si, Waters, Wilmslow, UK) with ACQUITY HSS T3, 1.8 µm, 2.1x100 mm, was performed for untargeted experiments in positive and negative electrospray ionization. The mass data was corrected during acquisition using lockspray of leucine encephalin ([M ± H] ± with m/z 556.2771 and negative mode [M - H] ± with m/z 554.2615). Pooled QC samples were injected at the beginning of the run, and in every 10th sample, to ensure accuracy an reproducibility during MS analysis. The mobile phase consisted of milli Q water with 0.1% formic acid, v/v (A) and methanol with 0.1% formic acid, v/v (B). The gradient was 0 min, 100% A; 16 min, 100% B; 20 min, 100% A, and 4 min, 100%. The flow rate was 0.4 mL/min. The column temperature was set at 40°C. The injection volume was 5 µL. The parameters were as follows: capillary voltage 3 kV; sample cone voltage 40 V; source temperature 150°C; desolvation temperature 500°C; desolvation gas flow 1000 L/h. Chromatograms were exported from MassLynxTM (V4.1) software and MarkerLynxTM XS to UNIFI Scientific Information System software. They were quantified by peak detection,

retention time alignment, and library matching for identifying chemical metabolites.

2.5 Statistical analysis

Statistically significant differences between days with COVID-19 groups were analyzed with related-samples Friedman's two-way ANOVA, Bonferroni test. A p-value of less than 0.05 was considered as statistically significant.

Multivariate analysis was used for data analysis. The data was divided into 4 groups; day 1 at 0 hr (baseline), day 1 at 2 hrs after AP administration, day 3, and day 5.

Principle component analysis (PCA) was performed for data overview and the orthogonal projection to latent structures-discriminant analysis (OPLS-DA) for different compounds between the groups. S-plot was used to visualize the significant biomarkers of the samples. The metabolite identification was performed with the human metabolomics database. Metaboanalyst 5.0 software was used for pathway analysis.

3. Results

3.1 General characteristics, clinical symptoms and signs after AP extract administration

Six patients with mild COVID-19 infection were recruited for this study. However, one was excluded due to a false positive test result (Fig. 1). The general characteristics are shown in Table 1. RT-PCR became negative on day 3 and day 5 after AP extract administration in all patients (Fig. 2a). We found that the symptom score of all patients was significantly decreased on day 5 (Fig. 2b). There was no significant difference of liver function test, renal function test and blood profile pre- and post-AP extract administration for all time points (Supplement S2).

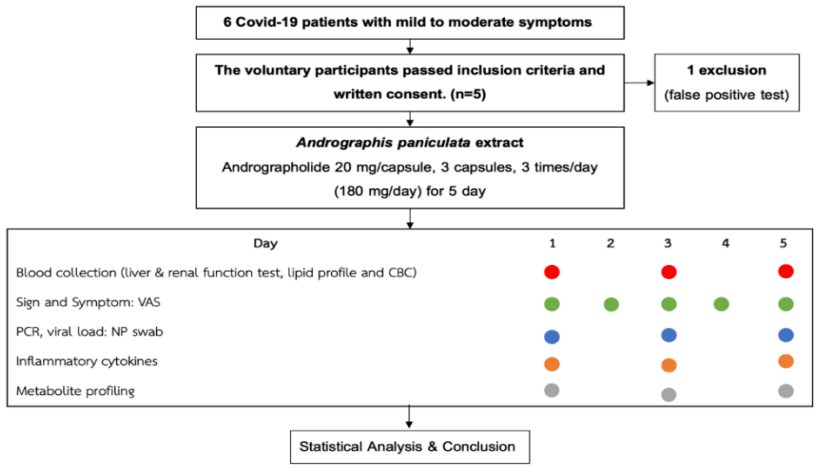

Fig. 1. Study flow and data analysis collection.

3.2 Effects of AP extract on pro-inflammatory cytokines, chemokines, and growth factors

To investigate the pro-inflammatory response of AP extract on five patients with mild COVID-19, the level of twenty-seven cytokines/ growth factors/ chemokines were measured by using Bio-Plex Pro Human Cytokine. The results revealed that the mean plasma cytokines linked to COVID-19. There were 6 adaptive immunity and pro-inflammatory cytokines including IL-2, IL-4, TNF-α, IFN-γ, IL-12, and IL-17, which significantly decreased to baseline (0 hours) (Fig. 3). The adaptive immunity and pro-inflammatory cytokines (IFN-γ) were found significantly decreased as early as three days until five days. The levels of pro-inflammatory cytokines (IL-2, IL-4, TNF-α, IL-12, IL-17)

were significantly decreased 3 days after treatment. However, these results had a trend to increase after treating with AP for five days, including the level of IL-7, GM-CSF, IP-10, G-CSF, and MCP-1. Although the plasma levels of adaptive immunity and cytokines gradually decreased with time, their levels tended to be higher in COVID-19 patient L-005 after treatment with AP for five days (IL-1β, IL-7, GM-CSF, G-CSF, MCP-1) (Fig. 3). These results possibly indicate that AP could affect some patients who have different metabolic functions. Additionally, we found that the effect of AP extract on other cytokines/ chemokines/ growth factors unrelated to COVID-19 were significantly decreased three days after AP treatment for (IL-9, RANTES, MIP-1b) and five days for (PDGF-bb) (data not shown).

Table 1. General characteristics of participants.

Factors	Total (n=5)	COVID-19 patients				
		No.1	No.2	No.3	No.4	No.5
Age (mean±SD)	33.4±12.2	26	21	27	46	47
Gender	3 Female :2 Male	F	F	F	M	M
Pulse (time/min)		84	90	102	90	76
Blood pressure (mmHg)		113/64	112/73	135/77	130/80	135/80
Respiratory rate (time/min)		18	18	22	18	n/a
Temperature (°C)		37	36.5	38	36.5	36.8
Blood group	2A :2O :1B	A	A	O	B	O
Body elements*	1fire: 2water :2 earth	Fire	Water	Earth	Earth	Water

Gender: F=female, M=male

*Body elements or Dhātu Chao Ruean are four elements in Thai traditional medicine, namely Fire, Water, Earth, and Wind.

3.3 Metabolite changes after AP extract administration

All samples and QC samples were analyzed by using PCA with variations of less than ± 2SD in both positive and negative ESI modes (data not shown). In this study, PCA of all detected metabolites did not show any substantial clustering before and after AP administration in both positive and negative modes (Supplement S3). After OPLS-DA analysis, they were separated among time points (Supplement S4).

S-plot was used to filter the metabolite biomarkers from OPLS-DA with p-values < 0.05. Forty-nine metabolites were identified as biomarkers. PCA was performed to observe alterations of identified metabolites before and after AP administration (Fig. 4). Data for day 1, before and 2 hours after AP extract administration, was separated from data for day 3 and day 5. The increase and decrease of metabolites over the time is shown in Fig. 5. The heatmap exhibits individualized pattern changes of metabolite levels. An increase in the metabolites related to inflammation such as Leukotriene D5 and prostaglandin D3 was found in patients no.1, no.2, and no.5 on day 3. However, on day 5, these metabolites were decreased in patient number 5 but remained the same in patients no.1 and no.2. Ten different pathways were found (Fig. 6). The major pathways related to AP extract were listed as follows: glycerophospholipid metabolism; phenylalanine, tyrosine and tryptophan biosynthesis; linoleic acid metabolism; valine, leucine and isoleucine

biosynthesis; alpha-linolenic acid metabolism;
and phenylalanine metabolism.

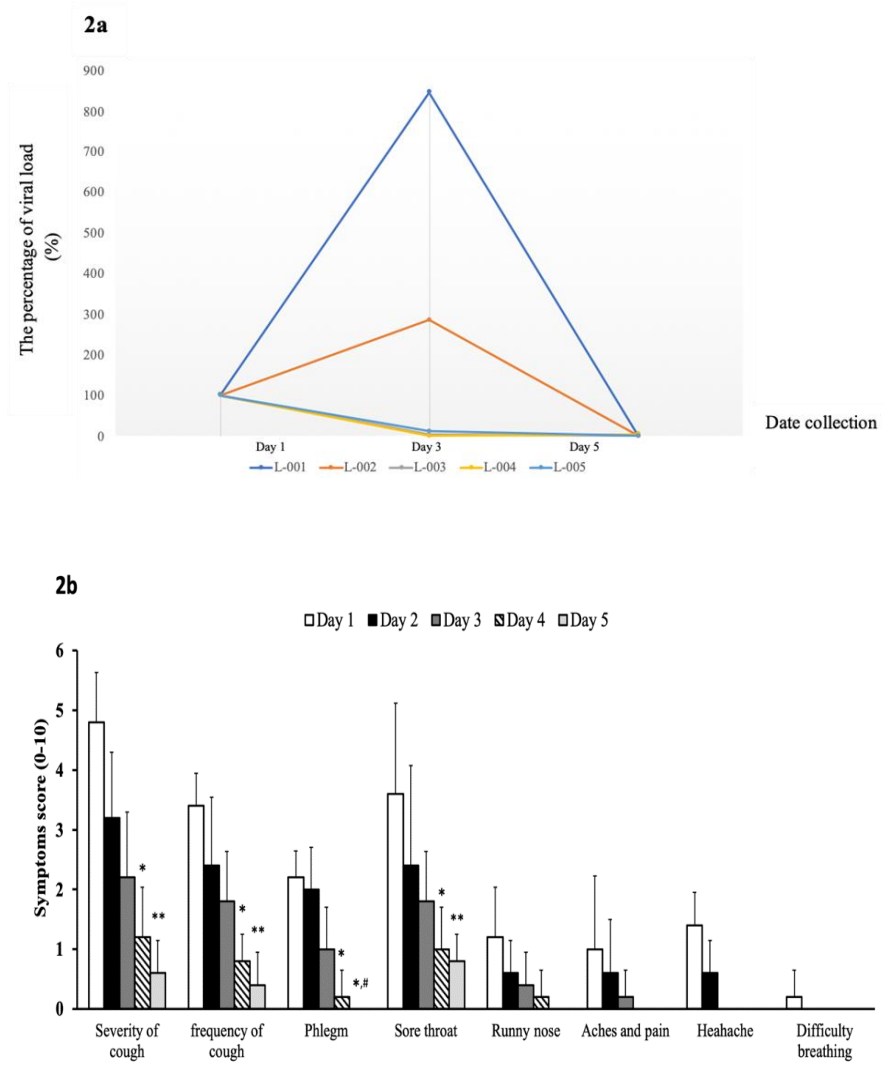

Fig. 2. RT-PCR and clinical symptoms after AP extract administration. (a) Line graph represents percentage of viral load by RT-PCR between day 1, day 3, and day 5 after AP extract administration. Vertical axis represents percentage of viral load (copies/ml) and horizontal axis represents the analytical days after AP extract administration. (b) The average symptoms score of the five COVID-19 patients with AP extract administration were measured from 0-10; 0=no symptom and 10=severe symptoms. Bars express Mean + SD for all time points analyzed by related-samples Friedman's two-way ANOVA, Bonferroni. *; $p < 0.05$, **; $p < 0.01$, as compared with baseline. #; $p < 0.05$, as compared with Day 2.

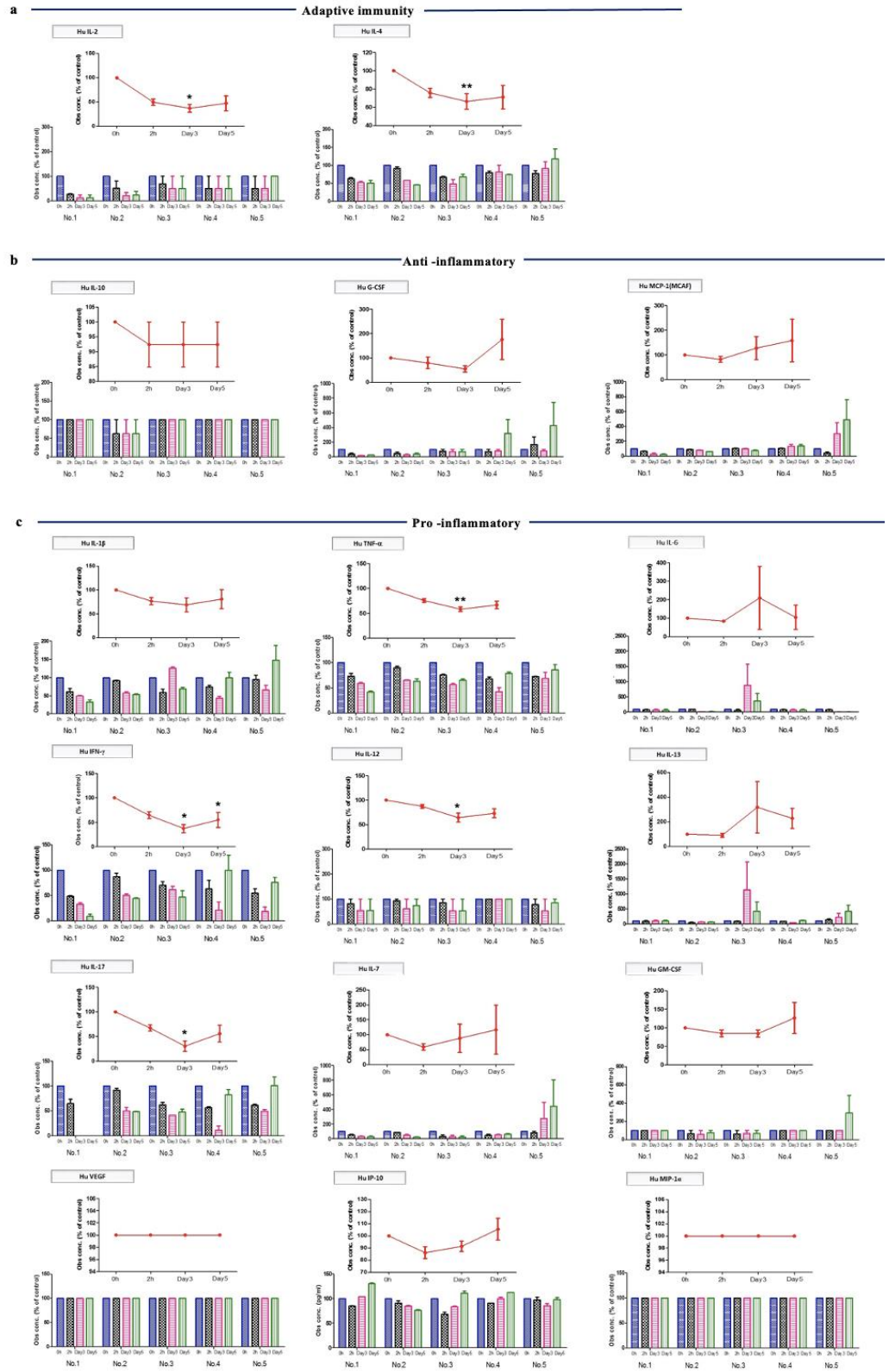
a
Adaptive immunity
Hu IL-2
Hu IL-4
b
Anti -inflammatory
Hu IL-10
Hu G-CSF
Hu MCP-1(MCAF)
c
Pro -inflammatory
Hu IL-1β
Hu TNF-α
Hu IL-6
Hu IFN-γ
Hu IL-12
Hu IL-13
Hu IL-17
Hu IL-7
Hu GM-CSF
Hu VEGF
Hu IP-10
Hu MIP-1α
Obs conc. (% of control)
0h
2h
Day3
Day5
No.1
No.2
No.3
No.4
No.5

Fig. 3. Cytokine level changes in the patients with mild COVID-19 infection after administration of a high dose of AP extract. The line graph shows the average cytokine level of the five volunteers, while the bar graph shows the individual cytokine levels of each patient. The level of adaptive immunity (IL-2, IL-4) and pro-inflammatory cytokines (TNF-α, IFN-γ, IL-12, IL-17) were significantly decreased in the patients. Graphs plotted as mean ± SEM. *; $p < 0.05$, **; $p < 0.01$, ***; $p < 0.001$ by related-samples Friedman's two-way ANOVA, Bonferroni, as compared with baseline.

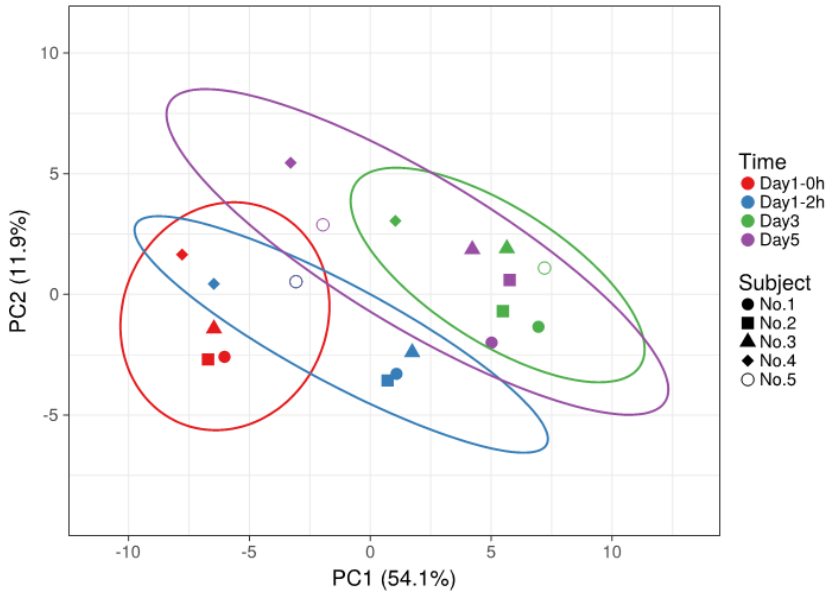

Fig. 4. PCA score plot of significant biomarker compounds analyzed by positive and negative ESI from plasma samples of five participants.

4. Discussion

This is the first study that has investigated the effects of *Andrographis paniculata* extract on patients with mild COVID-19 infection in Thailand. Firstly, the results demonstrated that a high dose of AP can decrease the clinical symptoms of the patients without affecting them adversely. Secondly, inflammatory cytokines related to COVID-19 infection were evaluated. Thirdly, the untargeted metabolomics study found inflammatory metabolite and nucleotide changes after treatment with AP extract.

Several studies have reported that adaptive immunity and pro-inflammatory cytokines become increased in patients with COVID-19 as the virus causes systemic inflammation. This mechanism plays a crucial role in immune regulation which in turn influences the severity of the disease in COVID-19 patients.[33, 34] This study showed that IL-2 and IL-4 was decreased after AP extract administration, indicative of the adaptive immunity related to JAK-STAT signaling pathways. Inhibition of IL-2/IL-2R in critical patients with COVID-19 pneumonia reduces CD8 ± T cells and lymphocytes through the JAK1(Janus kinase)-STAT5 (signal transducers and activators of transcription) pathway.[35] In this study, we found that AP extract can decrease the levels of six adaptive immunity and pro-inflammatory cytokines which are related to COVID-19 infection. Therefore, our study indicated that AP extract has anti-inflammatory effects reducing the severity of the disease in mild COVID-19 cases. However, further studies should be performed to confirm the anti-inflammatory and immuno-modulating effect of AP extract, especially andrographolide, against SARS-CoV-2.

The COVID-19 disease spectrum varies from asymptomatic to severe illness with a diverse range of phenotypes. Therefore, it is necessary to develop detection methods and to define the biomarkers. This study revealed lipid and amino acid metabolites related to inflammation, viral replication, and immune response in plasma of mild case COVID-19 patients. There is a study reporting that PGI_3, TXB_3, LXA_4, LXB_5 metabolites were increased along with an inflammatory lipid storm.[36] In a previous study of severe COVID-19 cases, the increased fatty acid level-related biosynthetic pathways were also enhanced in COVID-19 patients.[37] In this study, the plasma of mild case COVID-19 patients showed an increase in lipid metabolites such as phosphatidylcholines, phosphatidylethanolamines and phosphatidylserines on day 3 after AP extract administration. Lipid compo-sition is important to determine the viral capacity for entry and replication.[38] In addition, amino acids including tyrosine and L-phenylalanine metabolites were changed after treatment with AP extract. It usually presents in posttraumatic sepsis patients in whom the increase was related to immune response.[39] This may indicate a preference for immunomodulation in COVID-19 patients.

When AP extract containing andro-grapholide (180 mg/day) was given to the patients with RT-PCR positive Covid-19 within 72 hours for 5 days, they recovered within 3-5 days after treatment. As no adverse drug reaction nor abnormal blood chemical profile was seen after 5 days of AP exact administration, the dosage of this study is safe to use. Interestingly, most of the metabolites of patients no.1 and no.2 were increased on day 3, as compared to others. This may be due to increased viral load on day 3.

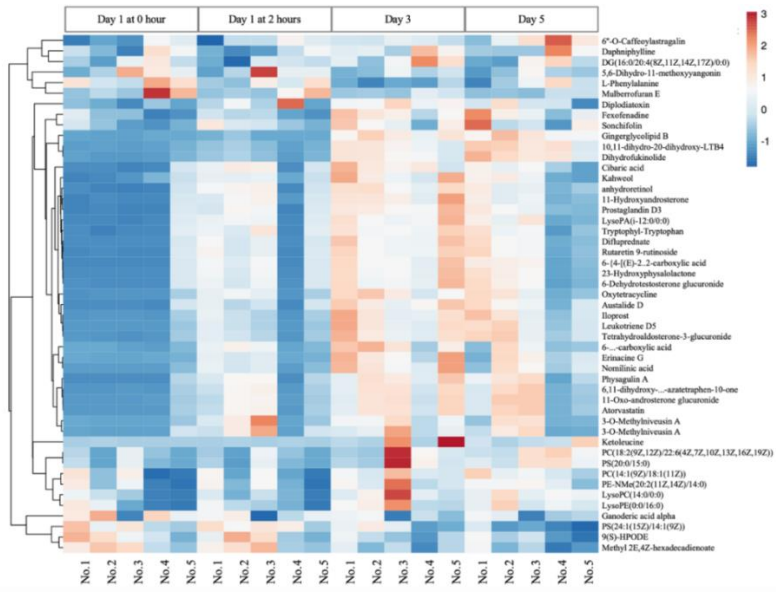

Fig. 5. Heat maps of the significantly different metabolite levels at each time point. X-axis represents the individual sample. Y-axis represents compounds. Red represents high normalized intensity, and blue represents low normalized intensity of the metabolites.

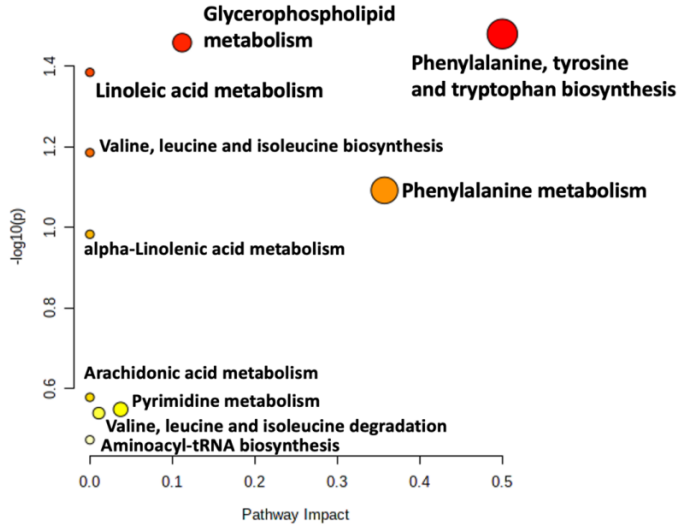

Fig. 6. Pathway analysis of 49 different metabolites of five participants at each time point.

In Thai traditional medicine, COVID-19 patients' 'body element' has been considered to be 'fire', which is alleviated by using 'cool tonic' Thai herbal medicine. However, a correlation between 'body elements' and patients' clinical response was not found. Therefore, AP may play a role to treat the clinical symptoms of COVID-19 patients as an alternative medicine to reduce the severity of the outbreak and reduce cost of importing medicines.

In our study, there were no control groups to compare clinical symptoms, pro-inflammatory cytokine and metabolite profiles. Clinical symptoms data of the patients before receiving AP extract was not available. Therefore, we cannot conclude whether the clinical symptoms were reduced due to the effects of AP extract or the self-healing mechanism of the patients. The number of patients in the study is not enough to determine the efficacy of AP extract for COVID-19 patients. Hence, a further study should be done with a control group and a larger number of patients.

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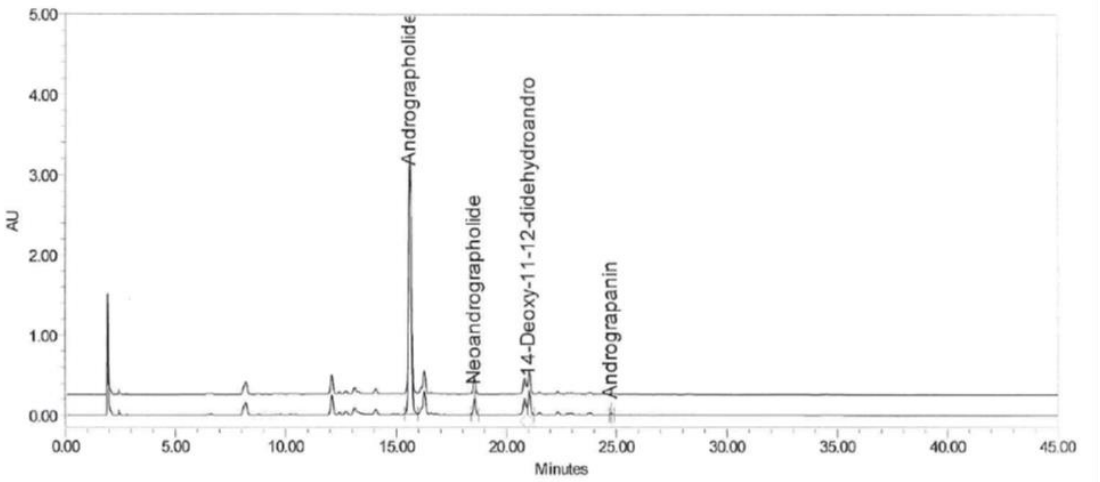
Competing interests

The authors declare that they have no competing interests.

Supplementary information.

(Mahajaroensiri S, Vannabhum M, . Leethong P, Wanaratna K, Inchai N, Chueawiang W, et al. Inflammatory cytokines and metabolite changes after high dose of *Andrographis paniculata* extract: A preliminary study in mild COVID-19 case patients. J Basic App Pharmacol. 2021;1(1):21-32.



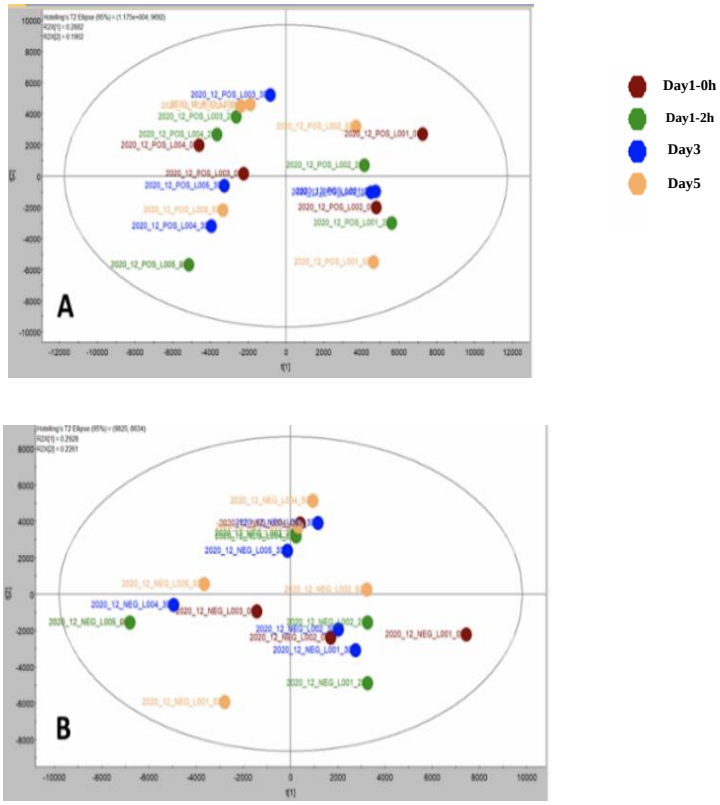
Supplement S1

Chromatogram of the AP extract capsule contents by HP-LC analysis for the quantitative and the qualitative identification of the active compounds. The retention times are 15.63 min for dndrographolide, 18.56 min for neoandrographolide, 21.05 min for 14-deoxy-11 12-didehydroandrographolide, and 24.78 min for andrograpanin.

Supplement S2
Biochemical profile before and after AP extract administration.

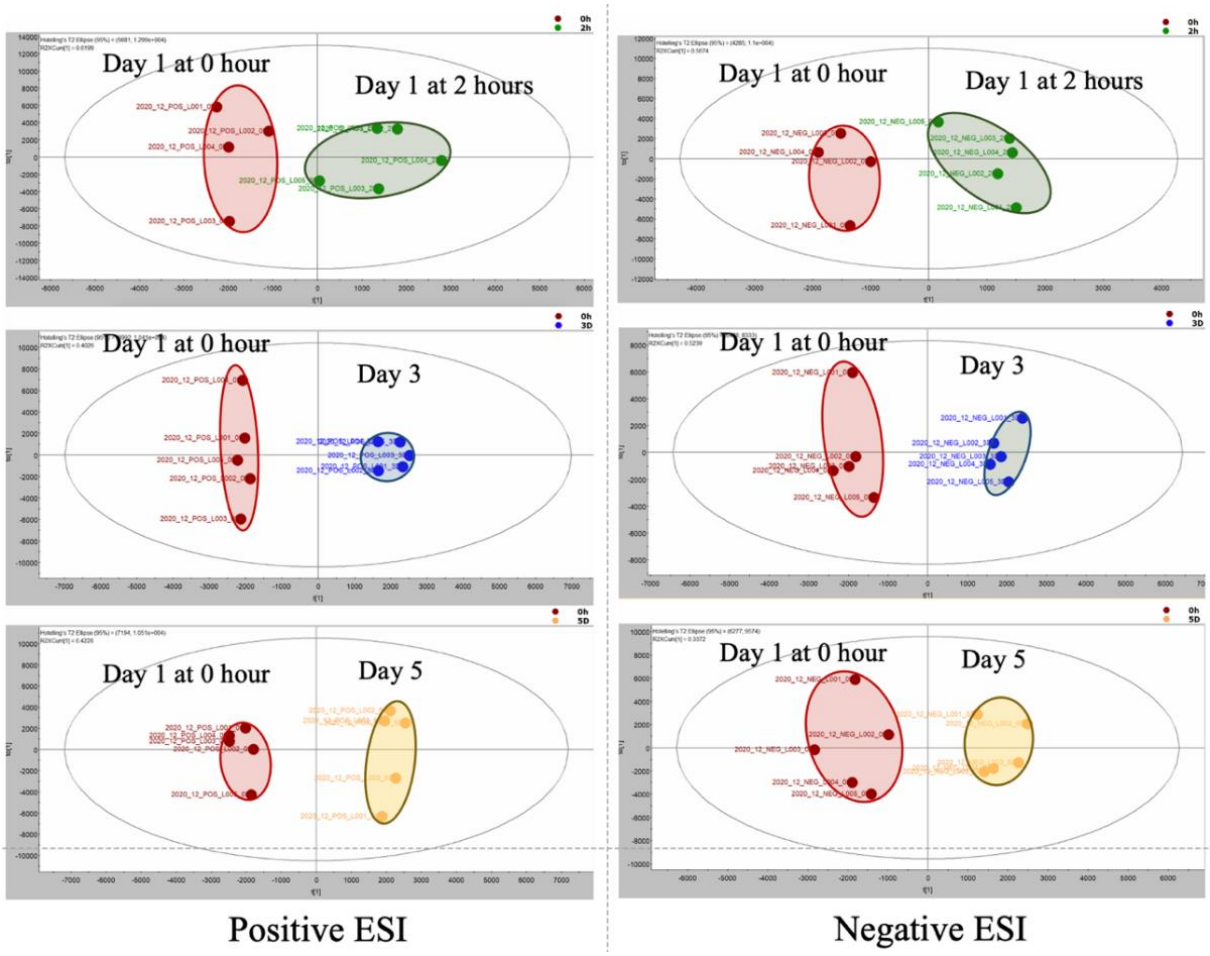
Factors	Day 1 (0 hr)	Day 1 (2 hrs)	Day 3	Day 5
ESR (mm/hr)	48.0±30.7	43.6±33.5	53.4±39.6	53.0±32.9
hsCRP (mg/L)	7.5±13.9	6.9±13.0	6.34±11.7	4.9±6.2
LDH (U/L)	293.4±65.8	343.2±118.4	354.2±100.1	315.6±59.0
Ferritin (ng/mL)	120.0±136.4	124±137.8	143.4±158.8	134.2±145.5
AST (U/L)	21.0±4.4	21.0±4.4	20.8±7.8	22.2±6.4
ALT (U/L)	14.0±8.1	14.0±6.3	14.0±7.9	27.4±26.3
ALP (U/L)	51.2±14.4	52.0±14.4	49.2±13.9	55.8±13.7
BUN (mg/dL)	11.5±2.2	11.12±2.4	12.44±3.5	12.2±3.2
Creatinine (mg/dL)	0.7±0.1	0.71±0.1	0.72±0.1	0.7±0.1
Total cholesterol (mg/dL)	156.2±23.6	154.6±23.6	152.0±22.4	155.0±21.4
Triglyceride (mg/dL)	78.4±6.0	63.2±18.1	78.8±28.6	115.0±37.9
LDL (mg/dL)	89.0±24.2	91±30.1	85.4±21.5	88.2±12.9
HDL (mg/dL)	59.0±14.0	60.2±10.2	61.2±10.6	55.6±15.9
RBC (x 10^3/µL)	4.6±0.3	4.70±0.3	4.81±0.4	4.8±0.6
WBC (x 10^3/µL)	8.2±2.6	7.78±2.8	7.80±2.6	6.8±2.1
Lymphocytes (x 10^3/µL)	32.8±11.0	26.58±7.5	30.56±11.0	27.9±4.7
Neutrophil (x 10^3/µL)	56.6±11.6	64.64±10.6	60.16±11.8	61.5±5.4
Platelet (x10^3/µL)	295.6±123.7	265.6±153.9	294.20±124.1	296.4±131.7
Hemoglobin (g/dL)	12.4±1.7	12.22±1.6	12.46±1.7	12.2±2.1
Hematocrit (%)	39.8±3.6	39.04±3.9	39.76±3.7	38.5±5.3

Paired t-test, $p<0.05$. Not significantly different before and after AP administration.



Supplement S3

PCA score plot of pre-dose and after AP extract administration (2 hours, Day 3, Day 5) A) PCA score plot of positive ESI, B) PCA score plot of negative ESI.



Supplement S4

The OPLS-DA plot and S-plot of reliability for group separation before and after AP extract administration (2 hour, 3 day, 5 day) A),B),C) Positive ESI, D),E),F) Negative ESI. The OPLS-DA S-plot of each variable that used a cut-off value for covariance of $p \geq |0.05|$ (magnitude) and $p(corr) \geq |0.1|$ (reliability), which indicates the most different compounds for each group. The 9 compounds were significantly different pre-dose as compared with after AP extract administration.

Supplement S5

Armpit temperature and oxygen saturation changes before and after AP extract administration.

No.	Day1	Day2	Day3	Day4	Day5	p-value
	Armpit temperature (°C)					
1	37.1±0.15	37.1±0.08	37.2±0.10	37.1±0.10	36.9±0.12	0.660
2	36.5±0.26	36.8±0.25	36.8±0.17	36.5±0.13	36.9±0.00	
3	37.0±0.29	37.1±0.19	37.1±0.12	37.0±0.34	36.8±0.19	
4	36.4±0.43	36.7±0.38	36.7±0.25	36.5±0.10	36.7±0.26	
5	36.7±0.21	36.5±0.22	36.5±0.41	36.7±0.32	36.7±0.25	
	Oxygen saturation (%)					
1	98.0±0.82	97.8±0.50	96.8±1.89	98.0±0.00	97.7±0.50	0.451
2	98.0±1.41	98.8±0.50	98.8±0.50	98.8±0.50	99.0±0.00	
3	97.5±1.00	97.8±0.50	98.5±0.58	97.8±0.50	98.3±0.50	
4	96.0±0.00	96.8±0.50	97.5±0.58	96.8±0.50	97.5±0.58	
5	97.5±0.58	96.8±0.96	97.5±0.58	97.8±0.50	98.3±0.50	

Related-samples Friedman's two-way ANOVA, Bonferroni.