Original Article

Anti-SARS-CoV-2 Activity and Inhibition of ACE2 and TMPRSS2 Expression of Ya Prasa Pro Yai, Ya Ha Rak and Ya Chanthalila Traditional Drug Formulas

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Abstract

Introduction and Objective: Coronavirus disease 2019 (COVID-19) is caused by the SARS-CoV-2. Some Thai traditional drug formulas have been prescribed in Thailand to alleviate symptoms caused by the virus. The objective of this study was to investigate the anti-SARS-CoV-2 properties of Ya Prasa Pro Yai (PSP), Ya Ha Rak (Y5R) and Ya Chanthalila (CTL) drug formulas used as antipyretic in Thai traditional medicine.

Methods: The drugs were extracted and screened for anti-SARS-CoV-2 activity using plaque reduction assay. The extracts were further investigated for ACE2 and TMPRSS2 enzymatic inhibition and gene expression assays of several host-target molecules in Calu-3 cells.

Results: PSP, Y5R, and CTL traditional drug formulas were found to have shown inhibitory activities against SARS-CoV-2 at 45.6%, 45.6% and 50.1%, respectively, at the concentration of 5,000 μg/mL. CTL also significantly inhibited (71.2%) the main human receptor ACE2 at the concentration of 2,000 μg/mL, which was higher than both PSP and Y5R extracts and demonstrated TMPRSS2 enzymatic inhibition in a dose-dependent manner. CTL also significantly down-regulated the expression of ACE2, but not TMPRSS2, PIKfyve and cathepsin L in Calu-3 cells.

Discussion: The Thai traditional drug formulas reported here provided anti-SARS-CoV-2 activities. Testing of the Anti-viral properties of the three preparations at 5 mg/mL did not reveal significant differences. However, CTL showed more anti-viral activity than others consistent with the previous study that the effect may be from *E. longifolia* Jack, one of the components of the formulation. CTL showed inhibition of ACE2 and TMPRSS2 enzymes in dose-dependent manner compared to the controls. Moreover, the CTL preparation also demonstrated a significant down-regulation of ACE2 gene expression on calu-3, human lung cell lines which are the mechanisms inhibiting the entry into the cells in the early phase of infection.

Conclusion and Recommendations: Ya Prasa Pro Yai (PSP), Ya Ha Rak (Y5R) and Ya Chanthalila (CTL)

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herbal drug formulas were effective against SARS-CoV-2. Ya Chanthalila should be especially reconsidered as the first-line drug in clinical trials, which can not only reduce fever, but also limit SARS-CoV-2 spreading in the patient.

Key words: anti-SARS-CoV-2 activity, ACE2; Ya Prasa Pro Yai, Ya Ha Rak, Ya Chanthalila

ฤทธิ์ต้าน SARS-CoV-2 และการต้านการแสดงออกของยีน ACE2 และ TMPRSS2 ของตำรับยาประสะเปราะใหญ่ ตำรับยาห้ารากและตำรับยาจันทลีลา

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บทคัดย่อ

บทนำและวัตถุประสงค์: โรคโคโรนาไวรัส 2019 หรือโควิด-19 เป็นโรคระบาดใหญ่ทั่วโลกมีสาเหตุจากการติดเชื้อไวรัส SARS-CoV-2 ในประเทศไทยได้มีการสนับสนุนให้มีการจ่ายยาตำรับแผนไทยเพื่อรักษาอาการป่วยที่เกิดขึ้น ดังนั้น การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาว่ายาตำรับแผนไทยบางตำรับที่มีสรรพคุณลดไข้ได้แก่ ตำรับยาประสะเปราะใหญ่ ตำรับยาห้ารากและตำรับยาจันทลีลาสามารถยับยั้งเชื้อไวรัสดังกล่าวได้หรือไม่

วิธีการศึกษา: การศึกษาโดยดำเนินการคัดกรองฤทธิ์ต้าน SARS-CoV-2 ในหลอดทดลองด้วยวิธี Plaque reduction assay สารสกัดที่มีฤทธิ์ต้านไวรัสจะถูกนำมาศึกษาปฏิกิริยายับยั้งเอนไซม์ ACE2 และTMPRSS2 รวมถึงทดสอบการแสดงออกของยีนในเซลล์ปอดเพาะเลี้ยงชนิด Calu-3

ผลการศึกษา: ผลการศึกษาพบว่าตำรับยาประสะเปราะใหญ่ ตำรับยาห้ารากและตำรับยาจันทลีลา มีฤทธิ์ต้าน SARS-CoV-2 ได้ร้อยละ 45.60, 45.60 และ 50.11 ตามลำดับ ที่ความเข้มข้น 5,000 μg /mL ตำรับยาจันทลีลายังยับยั้งเอนไซม์ ACE2 อย่างมีนัยสำคัญถึงร้อยละ 71.15 ที่ความเข้มข้น 2,000 μg/mL ซึ่งสูงกว่าตำรับยาประสะเปราะใหญ่และตำรับยาห้าราก ตำรับยาจันทลีลายังแสดงการยับยั้งเอนไซม์ ACE2 และ TMPRSS2 ในลักษณะความสัมพันธ์ที่ตอบสนองต่อขนาดยา ยับยั้งแสดงออกของยีน ACE2 ลงอย่างมีนัยสำคัญ แต่ไม่ยับยั้งการแสดงออกของยีน TMPRSS2, PIKfyve และ cathepsin L

อภิปรายผล: ผลการทดสอบแสดงให้เห็นถึงประสิทธิภาพการต้านเชื้อไวรัส SARS-CoV-2 ของตำรับยาแผนไทยทั้ง 3 ตำรับ คุณสมบัติต้านไวรัสของตำรับยาทั้ง 3 ชนิด ที่ 5 มก./มล. ไม่ได้มีความแตกต่างที่มีนัยสำคัญ อย่างไรก็ตาม ตำรับยาจันทลีลาแสดงฤทธิ์ต้านไวรัสสูงกว่ายาตำรับอื่น ๆ สอดคล้องกับการศึกษาก่อนหน้านี้ว่าฤทธิ์ต้านไวรัสอาจมาจากสมุนไพรรากปลาไหลเผือกซึ่งเป็นส่วนประกอบของสูตรตำรับยาจันทลีลา ตำรับยายับยั้งเอนไซม์ ACE2 และ TMPRSS2 ในลักษณะที่ขึ้นกับขนาดยาเมื่อเปรียบเทียบกับกลุ่มควบคุม นอกจากนี้ตำรับยาจันทลีลายังแสดงให้เห็นถึงการควบคุมการแสดงออกของยีน ACE2 ในเซลล์ปอดเพาะเลี้ยง (Calu-3)ลดลงอย่างมีนัยสำคัญ ซึ่งเป็นกลไกที่ยับยั้งการเข้าสู่เซลล์ในระยะแรกของการติดเชื้อ

ข้อสรุปและข้อเสนอแนะ: ตำรับยาประสะเปราะใหญ่ ตำรับยาห้ารากและตำรับยาจันทลีลามีฤทธิ์ต้าน SARS-CoV-2 ที่ดี และควรพิจารณาใช้ตำรับยาจันทลีลาในการทดสอบทางคลินิกเป็นยาลดไข้ในระยะแรกของการติดเชื้อ ซึ่งนอกจากจะช่วยลดอาการไข้แล้ว ยังอาจสามารถลดการแพร่กระจายของเชื้อไวรัสในผู้ป่วยได้

คำสำคัญ: ฤทธิ์ต้าน SARS-CoV-2, ACE2, ตำรับยาประสะเปราะใหญ่, ตำรับยาห้าราก, ตำรับยาจันทลีลา

Introduction and Objectives

Since the Coronavirus disease 2019 (COVID-19) outbreak in Wuhan, China in December 2019, more than 460 million people have been infected and 6 million have died.[1] The disease is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a coronavirus with a spike protein. The receptor-binding domain of the spike protein on the viral envelope of SARS-CoV-2 can directly and specifically bind to angiotensin-converting enzyme 2 (ACE2), a membrane-associated enzyme. The SARS-CoV-2 spike protein can be cleaved and activated by transmembrane proteases, including serine 2 (TMPRSS2) before being endocytosed into host cells. Once the virus enters the cell, the virus also needs the PIKfyve enzyme to form an endosome and transport virus particles in the cytoplasm. This process also needs cathepsin L protease, which is responsible for endosome maturity. The Inhibition of PIKfyve and cathepsin L protease has been shown to be effective against SARS-CoV-2, Ebola virus (EBOV) and African swine fever in the early and mid-stages of the infection.[2] Presently, the virus continues to mutate producing different strains including alpha (B.1.1.7), beta (B.1.351), gamma (P.1), delta (B.1.617.2) and omicron (B.1.1.529). Each mutation has an altered sequence at the receptor-binding domain (RBD), which interacts with ACE2 that directly affects the fitness and transmissibility of the virus.[3] Therefore, inhibition of ACE2 or TMPRSS2 and other host-target molecules could limit the transmission in the early phase of infection. ACE2 and TMPRSS2 have been studied and identified to be the main targets of SARS-CoV-2 inhibitors.[4-7] ACE2 and TMPRSS2 are mostly expressed not only in lung tissue but also in kidney, oesophagus, colon, heart, brain and other organs.[8-9] Patients infected with the virus might have symptoms such as fever, cough, shortness and difficulty in breathing and some complications of pneumonia, kidney failure or even death.[10]

In Thailand, some people have turned to using medicinal plants or alternative medicines to alleviate symptoms caused by the infection with most showing antipyretic properties or the inhibition of virus-caused disease. Traditional drug formulations in the National List of Essential Medicine including Ya Prasa Pro Yai, Ya Ha Rak and Ya Chanthalila formulations have been widely used for alleviating fever during the pandemic and have been prepared in several forms (capsules, dried powder and tablets). Formulations of these traditional drug have been published on the website of Thai Food and Drug Administration (Thai FDA).[11] Ya Prasa Pro Yai is specifically used to treat flu, stomach pain, flatulence and anorexia in children. The formulation con-

sists of 21 herbal plants, 50% (w/w) of which are PraoHom (*Kaempferia galanga* L.) which was reported to show various biological activities such as antimicrobial, antioxidant, amebicidal, analgesic, anti-inflammatory, anti-tuberculosis, anti-dengue, anti-nociceptive, anti-angiogenic and anti-cancer.[12] Ya Ha Rak (The five roots drug) which has gained recognition for its anti-pyretic properties which are recorded in the ancient Thai pharmacopeia. The formulation is composed of root powder from 5 plants including Ya-Nang (*Tiliacora triandra* (Colebr.) Diels), Konta (*Harrisonia perforata* (Blanco) Merr.), MaDueiChumphon (*Ficus racemosa* L.), ChingChi (*Capparis micracantha* DC.) and MaiTaoYayMom (*Clerodendrum indicum* (L.) Kuntze. The Ya Chanthalila formulation is composed of eight kinds of herbal plants including Koad-Kamao (*Atractylodes lancea* (Thunb) DC.), Koad-So (*Angelica dahurica* (Fisch.ex Hoffm.) Benth. et Hook. f.), Koad-Chulalumpa (*Artemisia vulgaris* Linn.), Chan-Daeng (*Dracaena loureiroi* Gagnep.), Chan-Khoaw (*Tarenna hoaensis* Pit.), Pla-Lai-Peark (*Eurycoma longifolia* Jack.), Ka-Dom (*Gymnopetalum cochinense* Kurz H.C.) and Bor-ra-pet (*Tinospora crispa* Miers ex. Hook. f.). The herbal plants in the formulation were previously reported to have biological activities including Anti-oxidant, anti-inflammatory, antinociceptive and antipyretic.[13]

K. galanga L. one of the major components of Ya Prasa Pro Yai has been reported to prevent quercetin binding to Mpro main protease and thus has potential as Sar-CoV-2 inhibitor.[14] YaNang leaves extract also has been reported to inhibited Delta variant of SARS-CoV-2 virus with 95.95% inhibition at 10 mg/mL.[15] and Koad-Chulalumpa, one of the herbal components in Chantaleela has been reported to inhibit Sar-CoV-2 with the EC_{50} below 0.1 mg/mL using the plaque reduction assay.[16] Taking these reports into account there seems to be evidence to suggest that Thai traditional herbal formulations may be useful for the treatment of Covid-19.[17-18]

The research presented here therefore aimed to investigate the anti-viral properties of Ya Prasa Pro Yai, Ya Ha Rak and Ya Chanthalila formulation extracts.

Methodology

1. Materials

1.1 Cell culture

Vero E6 cells, African green monkey kidney epithelial cells and Calu-3, lung epithelial cell line (ATCC, NY, USA), were grown in Eagle's minimum essential medium (EMEM) (Gibco, NY, USA) cell culture medium containing 2 mM L-glutamine, 0.1 mM non-essential amino acid, 1 mM sodium pyruvate and 10% fetal bovine serum at 37 °C in 5% CO_2 incubator.

1.2 Virus

SARS-CoV-2 virus (Delta variant/EPI_ISL_3797061) was obtained from a human nasopharyngeal swab. The virus was propagated in Vero E6 cells by three passages to establish a high-titer stock and stored at -80°C for use in all experiments. Virus titration as TCID50 titer/mL was performed. All experiments with live SARS-CoV-2 virus were performed at a certified biosafety level 3 facility, the National Institute of Health, Department of Medical Sciences, Thailand.

1.3 Standards and Drug formulations

B-eudesmol (> 98% HPLC grade) was form Merck KGaA (Darmstadt, Germany). Ya Prasa Pro Yai and Ya Ha Rak formulations were kindly gifted by Mr. Kaisee Limprasert, Thai Traditional Medicine (พทว.13150 พท.ภ. 13761 พท.ผ. 2001 บป.20). Herbal formulations were produced using the traditional household medicine formulation (2018) as listed in the National List of Essential Medicines. (Herbal medicine list, 2012); lot July 2021. The Ya Chanthalila formulation was purchased from Vejpong Pharmacy (HOCK AN TANG) company limited (Thailand); lot July 2021.

2. Methods

2.1 Extract production

Total 300 g of powdered plant was refluxed with distilled water. The resultant solutions were then filtered and further extracted with distilled water two times consecutively. The aqueous extract was then pooled, concentrated and dried by rotary evaporation yielding PSP, Y5R and CTL extracts. The dried extracts were collected and kept in a light-protected bottle at -20°C until used.

The extracts were also analyzed for their phytochemical compounds for quality control using Thin layer chromatography (TLC) using reverse phase, RP-18 plates (Merck KGaA, Darmstadt, Germany) as the stationary phase and the mixture of n-hexane: acetone: ethyl acetate (70:20:5 v/v) as the mobile phase. The drugs were monitored at 254 nm (A), 366 nm (B) and were sprayed with anisaldehyde sulphuric acid reagent. β-eudesmol was used as a standard. The plant extracts and the quality control data were conducted at the Herbal Quality Assurance Center, Medicinal Plant Research Institute, Department of Medical Sciences.

2.2 MTT assay

The cells were seeded at 5×10^3 cells in 100 μL medium per well of 96-well plates and left in the incubator for 24 hours. The medium was removed and the cells were treated with eight different concentrations of each compound in triplicate and then incubated for 48 hours. The medium was replaced with 200 μL MTT reagent and then continued to incubate for 4 hours. The MTT solution was discarded and 200 μL DMSO was added to each well to

dissolve the purple formazan product. The absorbance of the formazan product of viable cells was read using the microplate reader at 570 nm. The background absorbance was reduced by the blank and % viability was calculated compared to the control.

2.3 Anti-SARS-CoV-2 activity using plaque reduction assay

Vero cells were seeded at 3.5x105 cells in 3 mL medium per well of 6-well plates and left in the incubator for 24 hours to present in monolayer. The extracts were pre-incubated with SARS-CoV-2 at 37°C for 1 hour before transferring 200 μL of the solution with virus particles onto the monolayer of vero E6 cells. Viral adsorption was allowed for 1 hour in the CO_2 incubator. The cells were then washed with fresh medium to remove both the unbound viral particles and the extract/compound. Total 3 mL of medium was then added to the wells. The semi-solid medium was allowed to be set and all plates were placed in the incubator for 7 days. The overlaid medium was discarded and cells were fixed with 5% formaldehyde and then stained with 0.5% (w/v) crystal violet. The excess colour was washed with tap water. The plaques were counted and % inhibition was calculated compared to the controls (without the compound).[19] All extracts were used at previously determined non-toxic concentrations.

2.4 ACE2 inhibition assay

Angiotensin II converting enzyme (ACE2) activity was determined using the angiotensin II converting enzyme (ACE2) activity kit (Abcam, MA, USA) and conducted at the Toxicology Laboratory, Department of Medical Sciences. The enzyme solution and reagents were prepared following the procedure described in the manufacturer's protocol. The plant extracts were diluted with water at the highest concentration, which was not toxic to Calu-3 cells. A total of 48 μL of ACE2 assay buffer was mixed with 2 μL diluted ACE2 enzyme solution and then the solution was added to the diluted extracts or MLN4760 standard inhibitor (the background and enzyme controls were also tested). The mixed solutions were placed at room temperature for 15 min. A Total of 40 μL of ACE2 substrate mix was then added to the wells and immediately placed in spectrofluorometer for measurement (Ex/Em = 320/420 nm) in kinetic mode for 1 hour. The relative inhibition activity (%) of the sample was calculated using a relative fluorescence unit (RFU) compared to enzyme control.

2.5 TMPRSS2 Fluorogenic inhibition assay

The activity of the TMPRSS2 enzymes was determine using the TMPRSS2 Fluorogenic Assay Kit (BPS Bioscience, CA, USA) and conducted at the Toxicology Laboratory, Department of Medical Sciences. The enzyme

solution and reagents were prepared following the procedure described in the manufacturer's protocol. The plant extracts were diluted with water at the highest concentration, which was not toxic to Calu-3 cells and then diluted to lower 2 concentrations. A Total of 30 μL of TMPRSS2 enzyme at a concentration of 5 ng/μL in 1x TMPRSS2 Assay Buffer was added to 10 μL of diluted extracts or 10 μM camostat standard inhibitor (the background and enzyme controls were also tested). The mixed solutions were placed at room temperature for 30 min. A total of 10 μL of TMPRSS2 fluorogenic substrate mix (50 μM) was then added to all wells and placed at room temperature for 10 min whilst being protected from light. The plate was immediately placed in a spectrofluorometer and the fluorescent signal measured (Ex/Em = 383/455 nm) in kinetic mode for 30 min. The relative inhibition activity (%) of the sample was calculated using a relative fluorescence unit (RFU) compared to enzyme control.

2.6 Gene expression using real-time reverse-transcription polymerase chain reaction (RT-PCR)

Calu-3 cells were seeded at 5×10^5 cells/well in 6-well plates for 24 hours, then treated with the compounds or extracts at concentrations that were not toxic to the cells for 24 hours. Total RNA of each sample was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany). According to the manufacturer's protocol, RNA was re-suspended in 30 μL of nuclease-free water and the products ran on agarose gels to check the quality of the RNA. cDNA was synthesized using QuantiTect Rev. Transcription Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. Briefly, 2-μg template RNA was added to the reverse-transcription master mix and then the samples tubes were incubated at 42 °C for 15 min. The cDNA samples were tested in triplicate with quantitative PCR using a QuantiTect SYBR Green PCR Reagents kit (Qiagen, Valencia, CA, USA). A teetotal of 2 μL of each sample was mixed with SYBR Green PCR Master Mix and 10x QuantiTect Primers (Qiagen, Valencia, CA, USA), then performed the real-time PCR (RT-qPCR) followed the manufacturer's protocol in Thermal cycler (PCR) (Analytik Jena GmbH, Valencia, Jena, Germany). mRNA ratios relative to the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene were calculated for the standardization of gene expression levels. A melting curve analysis was also performed to verify the specificity and identity of PCR products. For selected genes, the data were analyzed using the equation described by Livak and Schmittgen[20] as follows: the amount of target $=2^{-\partial\partial Ct}$. The average ∂Ct from the untreated cells is a calibrator for each gene tested. This assay was conducted at Toxicology laboratory, Medicinal Plant Research Institute, Department of Medical

Sciences.

2.7 Statistical analysis

The total percentage of plaque reduction assay was expressed as the mean of duplicated experiments. The percentage of relative ACE2 and TMPRSS2 inhibition and relative quantity of mRNA expression was expressed as mean ± SD (n = 3) and statistical differences were analyzed using ANOVA and Kruskal-Wallis multiple comparison (GraphPad Prism 8.0.1).

Results

A total of 300 g PSP, Y5R and CTL yielded 9.30 g (3. 10% w/w), 5.70 g (1.9% w/w), and 51.50 g (17.10% w/w), respectively. From the result of the thin layer chromatograph (TLC) analysis, it was found that β-eudesmol, a monoterpene, had the retention factor (Rf) of 0.8. There were other spots in dark-blue and yellow colour in PSP, some yellowish spots in Y5R and less yellow CTL. Moreover, there were several blue spots in PSP and Y5R at Rf 0.8 and 0.5 witnessed at along-waved at 366 nm ultraviolet light (see in Figure 1 B). However, no spots were found at short-waved at 254 nm ultraviolet light (see in Figure 1 A).

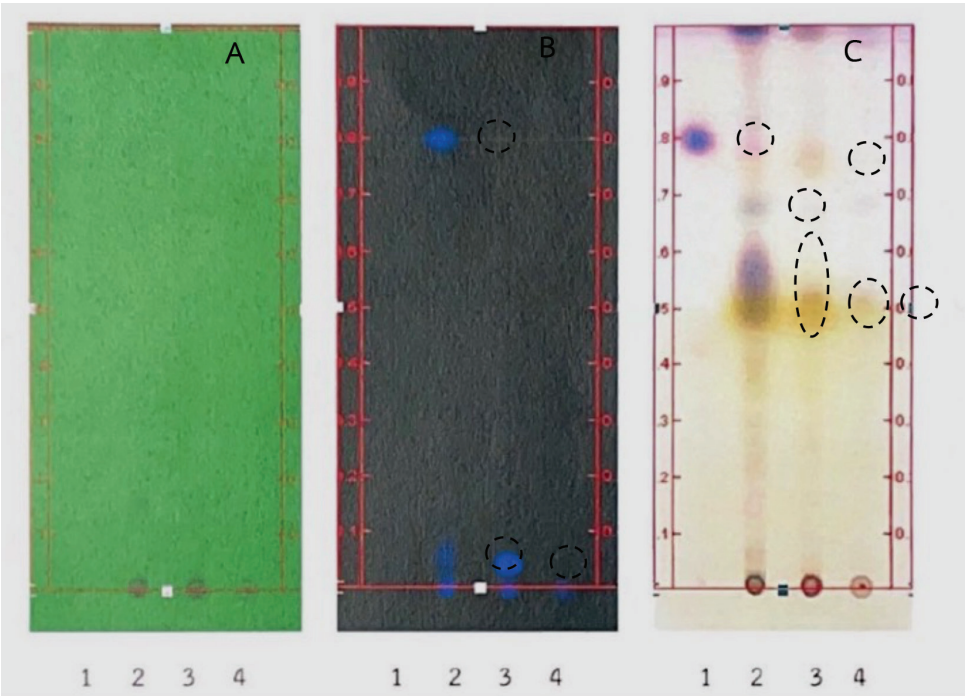
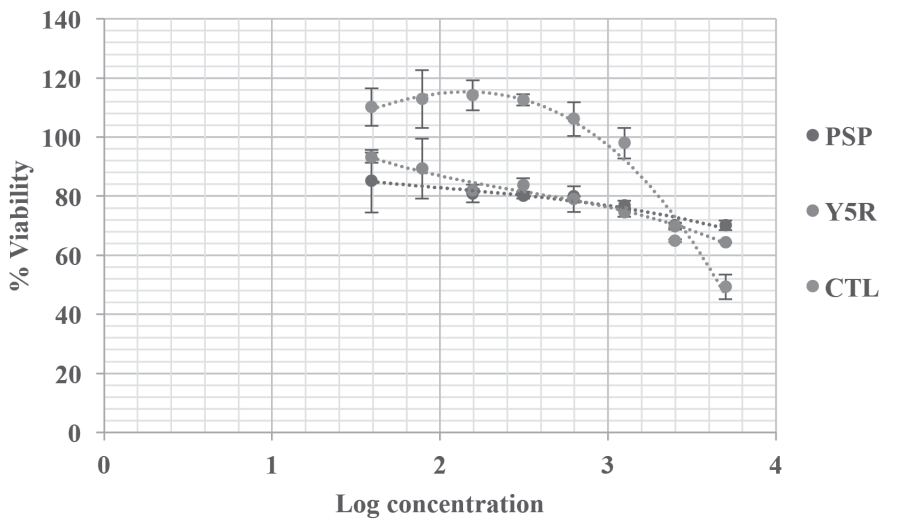


Figure 1 Thin layer chromatography of methanol extract of Ya Prasa Pro Yai (2), Ya Ha Rak (3), Ya Chan-thalila (4) drug formulations compared to β-eudesmol (1), using n-hexane: acetone: ethyl acetate (70:20:5 v/v) as mobile phases. The drugs were monitored at 254 nm (A), 366 nm (B) and were sprayed with anisaldehyde sulphuric acid reagent (C)

The ability of the different formulations to reduce the pre-entry phase of the SARS-CoV-2 virus was evaluated. Vero E6 cells were used as host cells for the virus. The drugs were evaluated at a concentration of 5.00 mg/mL, which was not toxic to the cells. The percentage of the plaque reduction was calculated compared with the virus controls. The CTL showed the highest plaque reduction of 50.11% while PSP and Y5R gave the lower percentage of the plaque reduction of 45.60%. The anti-viral activity of the three drug formulations is shown in Table 1.

Table 1 Anti-viral activities of Ya Prasa Pro Yai (PSP), Ya Ha Rak (Y5R), Ya Chanthalila (CTL). The data were expressed as the mean, n = 2.

Herbal extracts/compounds	The concentration tested (mg/mL)	% Plaque reduction
PSP	5.00	45.60
Y5R	5.00	45.60
CTL	5.00	50.11



	CTL		PSP		Y5R	
	Log conc.	Conc.(μg/mL)	Log conc.	Conc.(μg/mL)	Log conc.	Conc.(μg/mL)
IC_{50}	3.66	4597.27	4.63	42695.72	4.15	14068.70
IC_{20}	3.30	1995.50	2.54	346.96	2.66	453.74
IC_{10}	3.14	1377.86	0.81	6.50	1.77	49.56

Figure 2 Cytotoxicity of CTL, PSP and Y5R to calu-3 cell line. The concentrations were calculated from the non-linear regression as the inhibitory concentration (IC) at 50% (IC_{50}), 20% (IC_{20}) and 10% (IC_{10}).

The cytotoxicity of CTL, PSP and Y5R were evaluated using MTT assay. The non-linear regression was generated between log concentration and % viability. PSP gave the inhibitory concentration (IC) at 50% (IC_{50}) 20% (IC_{20}) and 10% (IC_{10}) of 42695.72, 346.96 and 6.50 μg/mL, respectively. Y5R gave the IC_{50}, IC_{20} and IC_{10} of 14068.70, 453.74 and 49.56 μg/mL, respectively. Whereas, CLT gave the lower cytotoxicity at the IC_{50}, IC_{20} and IC_{10} of 4597.27, 1995.50 and 1377.86 μg/mL, respectively.

ACE2 enzymatic inhibition of the extracts was evaluated at approximate concentrations between IC_{10} and IC_{20} values. CTL demonstrated inhibition in a dose dependant manner whereas, PSP and Y5R showed lower inhibitory activity (roughly 15-20% at the concentration of 25 and 50 μg/mL). CTL also inhibited TMPRSS2 enzyme in a dose dependent manner ($p < 0.5$). The % relative inhibition was 53.82, 63.90 and 71.15 % at the doses of 1,000, 1,500 and 2,000 μg/mL, respectively. ACE2 and TMPRSS2 enzymatic inhibition at different concentrations of PSP, Y5R and CTL were shown in Figure 3 and Figure 4.

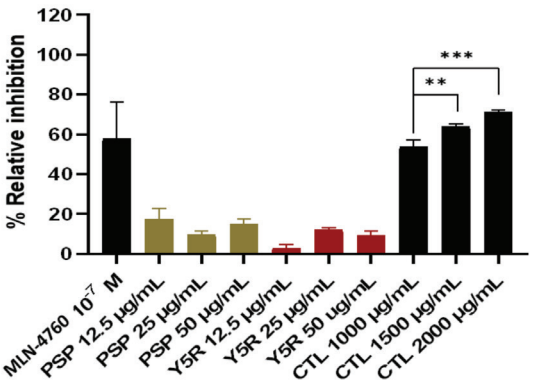


Figure 3 ACE2 enzymatic inhibitory activity at the highest concentrations which were not toxic to calu-3 cells of MLN4760 standard inhibitor, PSP, Y5R and CTL. Data is expressed as mean ± SD, n = 3. Statistical analysis was carried out using One-Way analysis of variance (non-parametric test) followed by Kruskal-Wallis multiple comparisons (**$p < 0.01$ and ***$p < 0.001$).

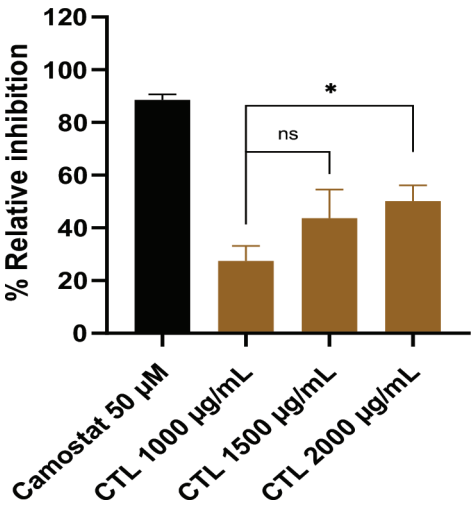


Figure 4 TMPRSS2 enzymatic inhibitory activity at the highest concentrations which were not toxic to calu-3 cells of camostat standard inhibitor, PSP, Y5R and CTL. Data is expressed as mean ± SD, n = 3. Statistical analysis was carried out using One-Way analysis of variance followed by Kruskal-Wallis multiple comparisons (*$p < 0.5$).

Gene expression assay of four main genes for viral entry and progression were analysed. Lung cells were treated with CTL at concentrations of 1000, 1500 and 2000 μg/mL. ACE2 gene expression was down-regulated compared to the untreated control and demonstrated a dose-response relationship. TMPRSS2 and PIKfyve demonstrated inhibition at the doses of 1,000 and 1,500 μg/mL, but not in dose-response manner. In contrast, the expression of cathepsin L was up-regulated at the doses of 1,000 and 1,500 μg/mL. The mRNA expression data of ACE2, TMPRSS2, PIKfyve and cathepsin L in calu-3 cells treated with different concentrations of CTL are shown in Figure 5.

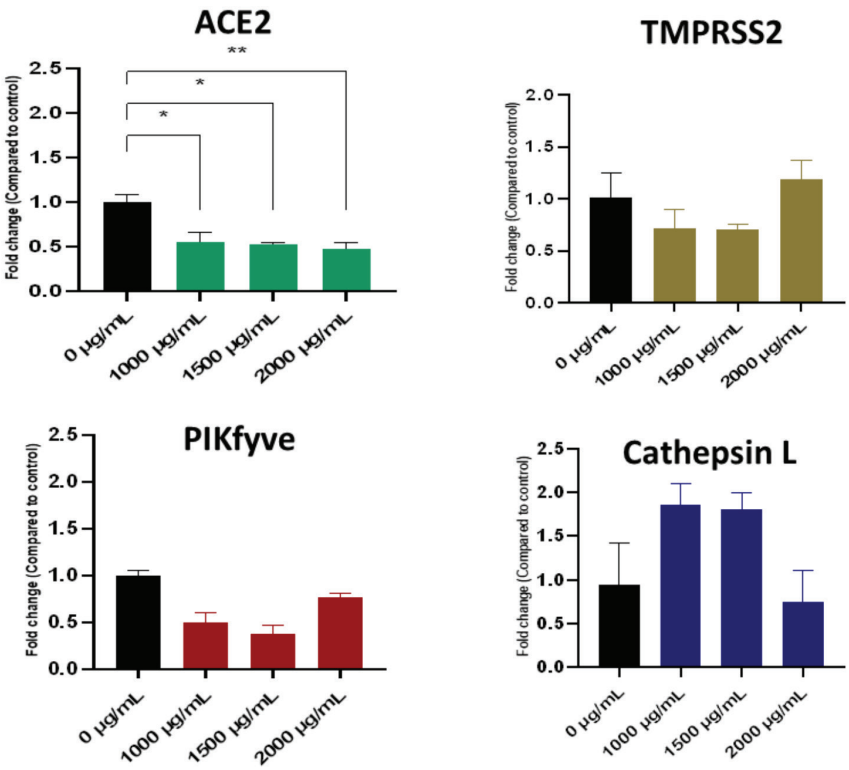


Figure 5 Relative quantity of mRNA expression of ACE2, TMPRSS2, PIKfyve and cathepsin L in calu-3 cells treated with CTL at 1,000, 1,500 and 2,000 μg/mL versus control data set for 24 h. Data is expressed as mean ± SD, n = 3. Statistical analysis was carried out using One-Way analysis of variance followed by Kruskal-Wallis multiple comparisons (*$p < 0.5$ and **$p < 0.01$).

Discussion

Ya Prasa PraoYai, Ya Ha Rak and Ya Chanthalila drug formulations which are traditionally used to treat flu due to their antipyretic properties were screened for anti-SARS-CoV-2 activity. The aqueous extract of PSP, CTL and Y5R are reported here for the first time to pose anti- SARS-CoV-2 activity. The three preparations used in this study are similar to the types of preparations used in Thai traditional medicine which are made of various medicinal plants.[11] *K. galanga* L. which is the main component in PSP, is now reported to include phytochemchemicals such as terpenoids, phenolics, cyclic dipeptides, diarylheptanoids, flavonoids, polysaccharides, and essential oils.[21] These phytochemicals were mostly presented in blue and yellow to anisaldehyde sulphuric acid reagent in contrast to Y5R and CTL which mostly presented yellow spots indicating the presence of different phytochemicals.

Ya Prasa Pro Yai preparation is normally used to treat gastrointestinal tract symptoms, e.g., stomach pain, flatulence and anorexia[18] because the main herbal component is *K. galanga* L. or PraoHom in Thai. PraoHom is also found to be one of the main components in the YaKheawHom preparation, which is used to treat measles and herpes simplex virus.[18] Ya Ha Rak formulation or the five roots drug formulation, is recommended by the Thai traditional TakKaSila pharmacopoeia to be the first-line drug formulation to treat flu according and was also recommended to alleviate symptoms during the Covid-19 infection. In contrast Ya Chanthalila has been effectively used during the recovery period following Covid-19 infection.[22] Testing of the Anti-viral properties of the three preparations at 5 mg/mL did not reveal significant differences. The main plant in the Ya Prasa Pro Yai preparationwas *K. galanga* L. which has been used to treat measles and herpes simplex virus infection.[21] Ya Ha Rak preparation consisted of five herbal roots in the same proportion. Some sources reported that chlorophyll derivatives and quercetin in *T. triandra* (Colebr.) Diels) could be potential therapeutic agents for treating COVID-19.[23] Meanwhile, Ya Chanthalila is made of different plants, some of which are used in Chinese traditional medicine and reported to be promising for SARS-CoV-2 treatment. *A. lancea* (Thunb) DC.) has been found using molecular docking analysis to lower binding energy with key proteins, suppresse cytokine storm and regulate some anti-viral pathways.[24] *A. vulgaris* L. has also been reported to demonstrate *in vitro* inhibition of FCoV or SARS-CoV-2 virus.[16] *D. loureiroi* Gagnep. and *T. hoaensis* Pit., the mixed ingredients in Kerra formulation have been found to inhibit the SARS-CoV-2 main protease and SARS-CoV-2 RdRp with IC_{50}

values of 49.91 ± 1.75 ng/mL and 36.23 ± 5.23 μg/mL, respectively.[25] Moreover, quassinoids, especially chaparrinone and eurycomalactone from *E. longifolia* Jack have been reported to inhibit SARS-CoV-2 with low IC_{50} values ranging from 0.32-0.51 μM.[26] We speculate that the *E. longifolia* Jack may be responsible for the anti- SARS-CoV-2 activity noted here in the CTL preparation.

CTL showed inhibition of ACE2 and TMPRSS2 enzymes in the dose-dependent manner compared to the controls with no inhibitory activity (0 % relative inhibition). Significantly CTL at the concentrations investigated showed inhibition of ACE2 and TMPRSS2 to the same extent as the standard inhibitor (see Figures 3 and 4). At the beginning of covid-19 pandemic, researchers targeted the ACE2 enzyme in attempt to block the entrance to human cells of SARS-COV-2 virus.[27] The CTL preparation also demonstrated inhibitory activity against infection using mRNA gene expression analysis on calu-3, human lung cell lines. The results were consistent with the ACE2 enzymatic assay and revealed a significant down-regulation of ACE2 gene expression.

In this study CTL has been shown to possess anti-SARS-CoV-2 activity. Preparations of CTL should be considered as the first-line drug to fight against SARS-CoV-2 in traditional clinics and hospitals.

Conclusion

From the results, the drug formulation extracts of CTL, PSP and Y5R showed inhibitory activity against SARS-CoV-2 at 45.60, 45.60 and 50.11 %, respectively at the concentration of 5000 μg/mL. The formulations also inhibited the main human receptor ACE2. CTL showed higher ACE2 inhibition than PSP and Y5R formulations. CTL also demonstrated TMPRSS2 enzymatic inhibition in the dose-response relationship manner. CTL significantly down-regulated the expression of ACE2 but not TMPRSS2, PIKfyve or cathepsin L in calu-3 which are involved in the pre-entry and early phases of infection. The Thai traditional drug formulations reported here provided anti- SARS-CoV-2 activity. The Ya Chanthalila formulation should be reconsidered as the first-line drug to fight against SARS-CoV-2 in clinical trials.

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