ประสิทธิผลของสมุนไพรไทยต้านท้องเสียในการยับยั้งช่องทางขนส่งคลอไรด์

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บทคัดย่อ

สะเดา (*Azadirachta indica Valeton*.), ทับทิม (*Punica granatum Linn*.), แค (*Sesbania grandiflora Linn*.), สมอไทย (*Terminalia chebula Retz*.), และขมิ้นชัน (*Curcuma longa Linn*.) เป็นสมุนไพรไทยที่ถูกใช้รักษาอาการท้องเสีย เมื่อนำสารสกัดสมุนไพรทั้งห้าชนิดมาศึกษาฤทธิ์ในการยับยั้งการไหลของคลอไรด์ผ่านช่องทางขนส่งโดยใช้เซลล์ต่อมไธรอยด์ของหนูที่มีการแสดงออกเฉพาะช่องทางขนส่งคลอไรด์มนุษย์ชนิด CFTR เท่านั้น สารสกัดหยาบจากน้ำและเอธานอลของเปลือกต้นสะเดา เปลือกผลทับทิม เปลือกต้นแค และเปลือกต้นสมอไทยมีฤทธิ์ยับยั้งการไหลของคลอไรด์ที่ความเข้มข้น 50, 50, 100 และ 200 ไมโครกรัม/มล. ตามลำดับ สารสกัดน้ำของเหง้าขมิ้นชันยับยั้งการไหลของคลอไรด์ที่ความเข้มข้น 200 ไมโครกรัม/มล. แต่สารสกัดเอธานอลเหง้าขมิ้นชันกระตุ้นการไหลของคลอไรด์ที่ความเข้มข้น 10 ไมโครกรัม/มล. เมื่อนำสารสกัดสามชนิด ได้แก่ เปลือกต้นสะเดา เปลือกผลทับทิม และเปลือกต้นแคมาศึกษาองค์ประกอบด้วยเทคนิครงคเลขผิวบาง พบสารกลุ่มแทนนินในสารสกัดจากพืชทั้งสามชนิด เปลือกผลทับทิมมีฤทธิ์ยับยั้งการไหลของคลอไรด์ในความเข้มข้นต่ำ เนื่องจากเปลือกผลทับทิมถูกใช้งานในรูปของเครื่องดื่มอยู่แล้ว จึงมีความปลอดภัยในการใช้เปลือกผลทับทิมเป็นยาจากอาหารในการรักษาอาการท้องเสียเฉียบพลันในขั้นต้น

คำสำคัญ: ต้านท้องเสีย สมุนไพร โปรตีนช่องทางขนส่งคลอไรด์

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Efficacy of antidiarrheal Thai medicinal plants in chloride channel inhibition

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Abstract

Antidiarrheal Thai medicinal plants, namely *Azadirachta indica Valeton*., *Punica granatum Linn.*, *Sesbania grandiflora Linn*., *Terminalia chebula Retz*., and *Curcuma longa Linn*. were investigated in the chloride channel inhibition model using Fisher rat thyroid cells stably expressing human CFTR chloride channel. The results demonstrated that *A. indica*, *P. granatum*, *S. grandiflora* and *T. chebula* extracts caused inhibition of CFTR-mediated chloride current at low dose starting from 50, 50, 100 and 200 µg/ml, respectively. This showed that these extracts' antidiarrheal activities stemmed partly from their CFTR chloride channel inhibition. *C. longa* aqueous solution showed an inhibitory effect at 200 µg/ml but its ethanolic extract showed chloride flux stimulating effect at 10 µg/ml. Extracts from *A. indica, P. granatum, S. grandiflora* were tested for their phytochemical groups using thin layer chromatography. All three extracts showed tannin. *P. granatum* peel with a low physiological dose is an existing ingredient in some beverages, thus it is safe to recommend *P. granatum* peel tea as a primary care medical food to treat acute secretory diarrhea.

Keywords: antidiarrheal, Thai medicinal plants, CFTR Cl^- channel

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Introduction

Cystic fibrosis transmembrane conductance regulator (CFTR) is a membrane protein and chloride channel in vertebrates. It is predominantly localized in kidney, airway, pancreas, intestine and testes. Mutation or dysregulation of CFTR underlies a large spectrum of diseases. Major pathologies of CFTR are cystic fibrosis and secretory diarrhea. Cystic fibrosis is the most common lethal genetic disease in Caucasians whereas secretory diarrhea remains a major health challenge worldwide[1,2].

In low and middle income countries, diarrheal diseases are responsible for 1.5 million deaths every year. It is mainly affecting children less than 5 years of age[3]. The majority of these deaths can be attributed to toxin mediated secretory diarrhea caused by infectious agents, such *as E. coli*, *V. cholerae* and Rotavirus[4]. Their enterotoxins up-regulates protein kinase A which is the prime regulator of the gene for CFTR[5]. CFTR has been implicated as the major Cl^- channel responsible for fluid secretion in secretory diarrhea caused by bacterial enterotoxins. Therefore, an inhibition of CFTR Cl^- channel is a potential drug target in cholera therapies and has attracted significant interests[6].

Medicinal plants are considered the basis for worldwide health preservation and healthcare. Plants and plant extracts have been used in different traditional medicine systems to treat various gastrointestinal ailments for so long. Natural products, i.e. hot tea, dark chocolate have been long used to treat diarrhea in humans. Some natural products possessed mechanism of action involving Cl^- channel inhibition[6]. Several Thai plants have historically been used for a traditional treatment of diarrhea including Sa-Dao: *Azadirachta indica Valeton*.[7], Tup-Tim: *Punica granatum Linn*.[7], Kae: *Sesbania grandiflora Linn*.[7], Samao-Thai: *Terminalia chebula Retz*.[7], and Kamin-Chan: *Curcuma longa Linn*.[7]. However, the cellular mechanism accounting for their antidiarrheal actions have never been verified.

Objective

To investigate the effect of five selected antidiarrheal Thai medicinal plants on CFTR Cl^- channel.

Materials and Methods

This research received Thammasat University, Faculty of Medicine Ethic Committee Clearance No. 153/2560.

Plant extract

In this study, crude extract of 5 plants; *P. granatum* (peel), *A. indica* (bark), *S. grandiflora* (bark), *T. chebula* (bark), and *C. longa* (rhizome) were tested for their abilities to inhibit CFTR-mediated Cl^- channel.

A. indica, S. grandiflora, C. longa and *S. grandiflora* were obtained from Srakaew province, Thailand in March 2007. *P. granatum* peel was obtained from Ayutthaya province in March 2007. All plant materials

were authenticated by Department of Thai Traditional Medicine, Faculty of Medicine, Thammasat University. After being cut and minced, the plants materials (bark, rhizome, and fruit peel) were dried at 50 °C for 7 days. Plant materials were then grounded. Crude extracts were prepared by aqueous and ethanolic extractions, respectively.

Aqueous extracts of five plant samples were prepared by placing 1 kg of each plant material in three liters of water, stirred, heated to boil and let them boil for 15 minutes. Aqueous solutions were then filtered through cheesecloth and paper filters, respectively. The aliquots were packaged and then freeze-dried at -50 °C. Crude extracts were stored in amber vials and kept at 4 °C.

Ethanolic extracts were prepared by macerated 1 kg of each plant materials in three liters of 95% ethanol at room temperature of 28 ℃ for 3 days. The extracts were filtered through cheesecloth and paper filters, then the volume concentrated by rotary evaporators. Crude extracts were stored in amber vials and kept at 4 °C.

Stock solutions of aqueous and ethanolic extracts of all five plants were made at 8 mg/ml in deionized water and DMSO, respectively, and kept at -20 °C. Working dilutions were made as a 50 µg/ml to be added to the chamber solution immediately at the starting of each assay.

Forskolin-induced chloride secretion in cell line

Fisher rat thyroid (FRT) cells stably expressing human CFTR (hCFTR) was cultured on Snapwell filters with 1 (cm^2) surface area (Corning-Costar, Acton, MA, U.S.A.) to resistance of >1,000 $\Omega.cm^{2,6}$.

Apical Cl^- current and short circuit current measurements

Apical buffer (130 mM $NaC_6H_{11}O_7$, 2.7 mM KCl, 1.5 mM KH_2PO_4, 2 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM Na-HEPES (pH 7.3) and 10 mM glucose) was prepared. Filters were mounted in a modified Ussing chamber and bathed with 4 ml apical buffer solution on both sides of the cell. For apical Cl^- current measurements in FRT cells, the basolateral hemichamber was filled with Ringer's buffer containing 130 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM Na-HEPES (pH 7.3) and 10 mM glucose. The basolateral membrane was permeablized with 250 µg/ml amphotericin B for 30 minutes before measurements. Solutions were bubbled with 95%O2,5% CO_2 and maintained at 37 °C. Apical Cl^-/short-circuit current was recorded using a DVC-1000 voltage-clamp (World Precision, Instruments, Sarasota, FL, U.S.A.) with Ag/AgCl electrode and 1 M KCl agar bridge.

Cl^- flux assay protocol

The apical chloride current was activated by adding 10 μM of forskolin to both apical and basolateral chambers. At the beginning of the work, the system was let run to stabilize, then forskolin was added to fully open Cl^- channel. Working solution was added at 50 μg/ml aliquot, then the current flow or current inhibition was observed and recorded. At the end of chloride flux decrease more working dilution was then added and observed. When current measured was approaching the beginning baseline, a 20 μM of CFTR (inh)-172 solution (Sigma-Aldrich, Singapore), a chloride channel inhibitor, was then added to terminate the test. Three tests were performed independently for each plant for both aqueous and ethanolic extracts. The percent short-circuit current of all tested samples were recorded.

Aqueous and ethanolic extracts of each plant were tested three times in separated occasions. Each experiment took 6 hours from start to finish. Mean percent inhibition and SEM were used to evaluate the data set.

Major phytochemical identification

The three most active extracts with CFTR Cl^- inhibiting activity were tested to identify major phytochemical groups using thin layer chromatography. Alkaloids, flavonoids, anthraquinone, coumarins, essential oils, tannins, saponins, phenolic and antioxidants were identified with group specific sprays[9].

Results

Effect of antidiarrheal Thai plants on CFTR in FRT cell

A. indica, P. granatum, S. grandiflora, T. chebula and *C. longa* aqueous and ethanolic extracts were tested in solution-bathing apical side of FRT cells. The Cl^- current inhibition percentages were plotted as shown in Figure 1-5. The aqueous and ethanolic extract of *A. indica, P. granatum* and *S. grandiflora* caused inhibition of CFTR-mediated Cl^- current at low dose starting from 50, 50 and 100 μg/ml whereas *T. chebula* started to show inhibitory effect at a much higher dose of 200 μg/ml (Figure 1-4). *C. longa* aqueous solution showed an inhibitory effect starting at 200 μg/ml but its ethanolic extract showed chloride flux stimulating effect at as low as 10 μg/ml (Figure 5). From these results, we decided to further investigate biologically active phytochemical components of the most three of active plant extracts, namely *A. indica, P. granatum* and *S. grandiflora*.

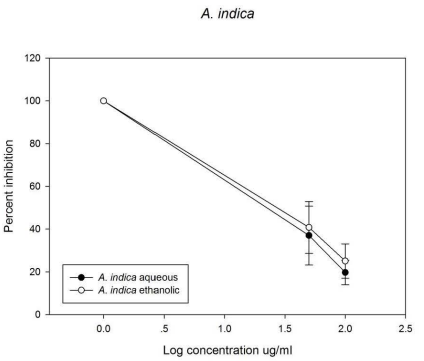

Figure 1 Effect of Sa-dao, *Azadirachta indica* Valeton. on CFTR-mediated Cl^- current inhibition model.

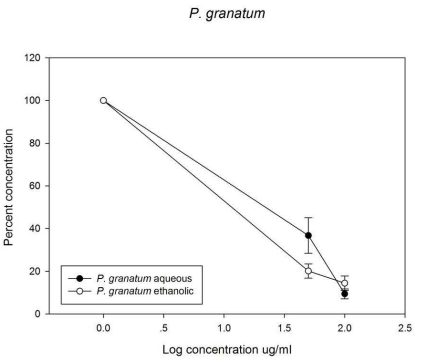

Figure 2 Effect of Tub-Tim, *Punica granatum* Linn. on CFTR-mediated Cl^- current inhibition model.

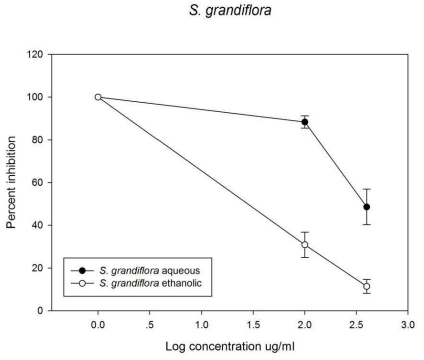

Figure 3 Effect of Kae, *Sesbania grandiflora* Linn. on CFTR-mediated Cl^- current inhibition model.

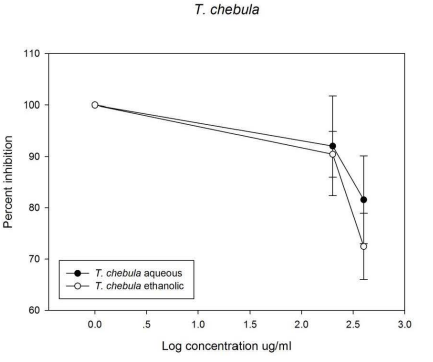

Figure 4 Effect of Samao-Thai, *Terminalia chebula* Retz. on CFTR-mediated Cl^- current inhibition model.

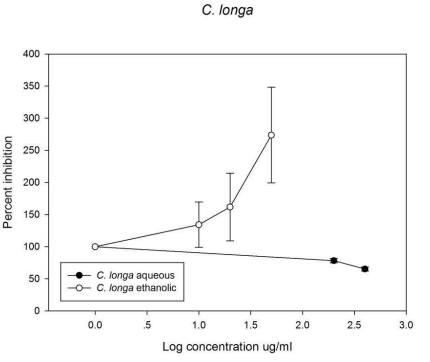

Figure 5 Effect of Kamin-Chan: *Curcuma longa* Linn. on CFTR-mediated Cl^- current inhibition model.

Major phytochemical screening

The most active three crude extracts, namely *S. grandiflora, A. indica* and *P. granatum* were subjected to be major compound group identification. The existence of various major compound groups was identified by positive spray reactions using compound specific sprays on thin layer chromatography product. The major compound groups found in positive inhibitory extracts are reported in Table 1.

Table 1 The composition of crude extracts the three most active plants, *S. grandiflora*, *A. indica* and *P. granatum* (W = water extract, E = ethanolic extract)

Phytochemical component	*S. grandiflora*		*A. indica*		*P. granatum*	
	W	E	W	E	W	E
Alkaloids		/				
Flavonoids	/	/	/	/	/	/
Anthraquinone	/	/		/		/
Coumarins	/	/	/	/	/	/
Essential oils		/	/	/	/	/
Tannins	/	/	/	/	/	/
Saponins	/	/	/	/	/	/
Phenolic		/		/		/
Antioxidants	/	/	/	/	/	/

S. grandiflora bark ethanolic extract showed all classes of major phytochemical groups, namely alkaloids, flavonoids, anthraquinone, coumarins, essential oils, tannins, saponins, phenolic and antioxidants. Similar results were presented in *A. indica* bark and *P. granatum* peel ethanolic extracts but no alkaloids were detected. Aqueous extracts of all three plants yielded fewer major compound groups, and no phenolic compound was detected.

Discussion

The conventional antidiarrheal treatment in Thailand up to now is based on two mechanisms; using antibiotic to kill the causative pathogens and using oral rehydration solution to replenish the lost fluid and electrolytes. According to the pathophysiology of diarrhea, the ideal antidiarrheal drug should have the ability to 1) decrease luminal osmolarity for osmotic diarrhea 2) decrease electrolyte secretion

and increase electrolyte absorption for secretory diarrhea and 3) decrease intestinal motility that causes a decreased transit time. Antibiotics are used for diarrheal patient who had enteric pathogen identified. However, many antidiarrheal drugs possess various adverse effects. The overuse of antibiotics had become the major factor of multi-drug resistant strains of microorganism. Herbal remedies are alternative treatment that possessed lesser adverse effects than the conventional drugs[10]. This study reported CFTR Cl^- channel inhibition effect of selected Thai plants which had long been used in Thailand pharmacopeia for their antidiarrheal efficacies. Extracts from *A. indica, P. granatum, S. grandiflora, T. chebula* and *C. longa* inhibited CFTR Cl^- channel in CFTR-expressing FRT cells at various concentrations. This showed that part of their antidiarrheal activities stemmed from their CFTR Cl^- channel inhibition.

P. granatum peel has long been used in Thai traditional medicine against diarrhea. Dried pulverized *P. granatum* peel is currently sold as a beverage item in the form of tea in online marketplace. As a result of this investigation, it is safe to recommend *P. granatum* peel tea to be used as a primary care medical food to treat acute secretory diarrhea when other medical help is not yet at hand.

The best performer, ethanolic extract of *P. granatum* peel, inhibited 80% of Cl^- flux at as low as 50 µg/ml while its aqueous counterpart showed 60% inhibition, as can be seen from Figure 2. It is outstanding that *A. indica, P. granatum* and *S. grandiflora* caused inhibition of CFTR-mediated Cl^- current at low dose starting from 50-100 µg/ml whereas *T. chebula* showed inhibitory effect at a much higher dose of 200 µg/ml (Figure 1-4). *C. longa* aqueous solution showed an inhibitory effect starting at 200 µg/ml but its ethanolic extract showed chloride flux stimulating effect at as low as 10 µg/ml. All of these plants have previously been reported to process antimicrobial effects[7]. *P. granatum* peel has been reported to have an antimicrobial effect against *Salmonella enterica* which is one of diarrheal causative agent[10]. Our result suggested that *P. granatum* peel exerted its antidiarrheal activity by inhibition of CFTR-mediated Cl^- current on top of its previously reported antimicrobial bioactivity.

The aqueous extract of *P. granatum* peels contained biologically active compound groups, namely tannins, alkaloids, and flavonoids that mediated its antidiarrheal property by inhibiting intestinal motility and intestinal fluid accumulation[11]. Literature in phytochemical screening of methanolic extract of rind of *P. granatum* fruit revealed the presence of flavonoids, alkaloids, tannins, glycosides. The antidiarrheal activity of the *P. granatum* peel extract in castor oil-induced and magnesium sulfate induced diarrheal mice was suspected to be derived from those phytoconstituents present[12]. In our study *P. granatum* aqueous extract was biologically active in inhibiting CFTR

Cl^- channel. This showed that part of its antidiarrheal activity stemmed from their CFTR Cl^- channel inhibition as well. It is not surprising to find that one plant extract can possess more than one mechanism of biological activity. Tannin and flavonoid seem to be the common chemical groups found among all biologically active diarrheal treatment reports using *P. granatum* peel. Both can be water soluble and can easily be extracted out when making tea from the fruit peel.

A. indica is one of the most respected trees among Indians. Various parts of the plant such as flower, leaf, bark and seed have been extensively used to treat various ailments since ancient times[13]. Literature reported that methonalic extract of *A. indica* leaves showed antisecretory activity on *Vibrio cholerae* induced fluid secretion in mouse intestine[14]. Both aqueous and ethanolic extracts of *A. indica* yield a good Cl^- flux inhibition activity in our study. Although over 300 components have been identified from this plant, only few have been thoroughly investigated. This is the first report of *A. indica* activity against CFTR Cl^- channel inhibition.

The antibacterial activity of *S. grandiflora* extracts investigated by micro-dilution method demonstrated that ethyl acetate fraction of *S. grandiflora* bark had a strong inhibitory activity against *S. aureus* with the MIC and MBC values less than 1 mg/ml[15]. Ethanolic extract of *S. grandiflora* leave had inhibitory effect against diarrheal causative agent *Burkholderia sp.*[16]. No report was found on verification of *S. grandiflora* usage and its mode of action against diarrhea to date. Our finding is the first to report its activity against CFTR Cl^- channel inhibition, showing another mechanism to verify the use of *S. grandiflora* as antidiarrheal treatment on top of its antimicrobial activity.

T. chebula exhibited many medicinal activities due to the presence of a large number of polyphenols, terpenes, anthocyanins, flavonoids, alkaloids and glycosides. Various parts of this plant had been traditionally used for the treatment of various ailments for human beings including diarrhea[17]. However, its inhibitory effect of CFTR-mediated chloride current was not impressively shown in the bark extract in present study. Fruit extract is recommended for further CFTR Cl^- channel inhibition investigation as it is a part often used in traditional medicine.

Learng Pid Samud recipe is a household remedy in the National List of Essential Medicines of Thailand, for the treatment of non-infectious diarrhea[18]. According to the applied Thai traditional medicine, this recipe is composed of fourteen kinds of herbal plants. *C. longa* is one of its main ingredients. Aqueous extract of *C. longa* showed mild Cl^- inhibition effect. *C. longa* was reported to have antidiarrheal effect on giardia diarrheal by improving intestinal mucosal damages induced by Giardia infection[19]. It also showed antidiarrheal effect on Escherichia coli by modulating gene

expression profiles in ileal mucosa of weaned pigs after an *E.coli* infection[20]. None of the previous works were tested on Cl^- channel module. It is quite a surprise to find that ethanolic extract of *C. longa* rhizome has an activating effect on Cl^- channel. *C. longa* has so many compounds with activities on many physiological systems. More work is needed to study the active compounds in our model and their activation pathways in details.

The use of tannins for treating gastrointestinal diseases can be dated back for a long time. Albumin tannate tablets have been used to treat diarrhea since 1900's. Tannins showed efficacies in clinical studies treatment of acute diarrhea[21]. Tannic acid was shown to protect against the adverse intestinal permeability changes during infection by improving the mucosal resistance[22]. Tannic acid was found to inhibit CFTR-dependent Cl^- secretion[23]. Further work is planned to derive the active compound of *P. granatum* peel from this CFTR Cl^- channel inhibition.

In 2012, Cesinex®, a mixture of at least nine compounds with various numbers of gallic acid groups attached to a central glucose or quinic acid core was tested to evaluate the efficacy and safety profile for treatment of diarrhea in pediatric and adult patients[24]. The tannic acid based medical food was shown to be effective in managing diarrhea and has a good safety profile. As a medical food, it can be used in combination with other prescriptions, oral rehydration solutions, or probiotic dietary supplements.

Conclusion

P. granatum, S. grandiflora and *A. indica* are prominent antidiarrheal Thai plants inhibiting CFTR Cl^- channel secretion. Depending on many pathophysiology of diarrhea, *P. granatum* peel showed CFTR Cl^- channel inhibition as an additional antidiarrheal mechanism from inhibiting intestinal motility previously reported in the literature. All plant extracts showed biologically active components with tannin component. The best performer was *P. granatum* peel extract. The aqueous extract of *P. granatum* peel as peel tea has high efficacy as demonstrated in CFTR-Cl^- channel inhibition and easy to use. It is safe to recommend *P. granatum* peel tea use as a primary care medical food to treat acute secretory diarrhea.

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