Phytochemical and Protein Extraction of Golden Shower Tree (*Cassia fistula*)

Patcharee Mongkolvai[1]*, Wilaiporn Insuwan[2], Jakkaphun Nanuam[3], Kamolrat Bunjan[4], Weerayut Phosai[4], Chutima Thanomsit[4], Amnuay Wattanakornsiri[5] and Paongpetch Phimchan[6]

[1]Department of Fisheries, Faculty of Natural Resource, Rajamangala University of Technology Isan Sakonnakorn Campus, Sakonnakorn 47160 Thailand

[2]Department of Science and Mathematics, Faculty of Agriculture and Technology, Rajamangala University of Technology Isan Surin Campus, Surin 32000 Thailand

[3]Program of Natural Resources and Environment, Faculty of Science and Social Sciences, Burapha University, Sakaeo 27160 Thailand

[4]Department of Fisheries, Faculty of Agriculture and Technology, Rajamangala University of Technology Surin Campus, Surin 32000 Thailand

[5]Program of Environmental Science, Faculty of Science and Technology, Surindra Rajabhat University, Surin 32000 Thailand

[6]Department of Plant science, Textile, and Design, Faculty of Agriculture and Technology, Rajamangala University of Technology Surin Campus, Surin 32000 Thailand

* Corresponding author. E-mail address: patcharee.mo@hotmail.com

Received: 13 January 2020; Revised: 15 June 2020; Accepted: 22 June 2020

Abstract

The objective of study was mainly to perform crude extracts from flowers and leaves of *Cassia fistula* by using two types of solvents: ethanol and hexane in order to reduce chemicals' or antibiotics' applications. After studying phytochemical substances by testing color development and sedimentation in both fresh and dried forms, we found that fresh leaves had higher phytochemical properties than flowers. There were 8 important properties found, including alkaloids, flavonoids, coumarin, metanin, flobatannins. turpenoids, steroids, and cardiac glycosides in the fresh leaves; on the other hand, we found 6 properties in fresh flowers, comprising alkaloids, flavonoids, coumarin, metanin, flobatannins, steroids, and cardiac glycosides. The fresh leaves were used in protein form identified by using SDS-PAGE technique, the important protein found having molecular weight between 23 to 80 kDa, consisting of seven bands: 80, 71, 52, 48, 40, 35, and 23 kDa. Moreover, their antibacterial activities were studied using disc diffusion method and dilution method and found that the crude extract had inhibitory activity on Gram positive bacteria, *Staphylococcus aureus,* and gram negative bacteria, *Pseudomonas aeruginosa* when compared with the antibiotic, chloramphenicol. The crude extract from fresh leaves showed inhibitory activity in *Staphylococcus aureus* higher than those from dried leaf extract. In the concentration of 3 mg / ml, the largest inhibitor zone observed was 12.67 ± 3.33 mm. For gram negative bacteria, the largest inhibitory zone found in the treatment of dried leaf extract with the concentration of 3 mg / ml was 12.33 ± 3.88 mm. In the study of antibacterial activity by dilution method, the extracts in all concentration levels could inhibit *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Based on these results, we concluded that *Cassia fistula* leaves can be used to perform as crude extracts and then applied for bacterial inhibition.

Keywords: Phytochemical, Golden shower tree, Protein extraction

Introduction

After some anti-biotics and agrochemicals were banned, agriculturists looked for other substances to substitute them in preventing and curing fish diseases. Potential substances gained interest are herbs because of their many advantages. For example, they are naturally available and can be grown. The agriculturists do not need to buy expensive antibiotics, meaning that they can be self-reliant without needing to lean on an antibiotic production company. Most herbs being recognized are safely applied to treat diseases in humans and

various animals for a long time. And, many herbs are scientifically confirmed as having antimicrobial activities such as bacteria, fungi, and viruses. Some herbs express antioxidant activities. For examples, they can reduce stress in fish, allowing fish to thrive and less likely to become infected. Moreover, they can stimulate immune (immunostimulation) in both humans and animals including fish (Chitmanat, 2013; Tebsun, Kronkpong, Rodloy, & Jintasataporn, 2016).

The chemicals found in many herbs have been proven and confirmed as having the abilities to inhibit microbial growth, stimulate the immune system, remove free radicals, reduce stress and prevent disease, such as alkaloids, flavonoids, phenolics, terpenoids, steroids and essential oils.

Cassia fistula is a tropical plant and widely found in Thailand. It has medicinal properties, as indicated in many reports about many phytochemicals extracted from fruits and seeds, having antibacterial activities such as beta-sitosterol, stigmasterol, ergosterol, betulinic acid, lupeol, fucosterol, and alpha-amyrin, as well as environmentaly friendly. It can inhibit ergosterol synthesis in the cell wall of Candida by at least 63.8% (Irshad et al., 2013). *Cassia fistula* leaf extracts by hydro-alcohol can inhibit gram-positive bacteria such as *Staphylococcus aureus* and *Streptococcus* spp., gram-negative such as *Escherichia coli* and *Pseudomonas aeruginosa*, and fungi such as *Aspergillus niger, Aspergillus clavatus* and *Candida albicans*. Moreover, Panda, Sujogya, and Mohanty (2011) found that *Cassia fistula* leaf extracts by petroleum ether, chloroform, ethanol, methanol and water inhibited gram-positive bacteria such as *Staphylococcus epidermidis, Bacillus licheniformis* and *Bacillus subtilis*.

The objective of this research were to study the basic phytochemicals of crude extracts, using hexane and ethanol, from the flowers and leaves of *Cassia fistula* and to investigate their anti-bactericidal activity for further application in agriculture and medicine.

Methods and Materials

Preparation of crude extracts of *Cassia fistula*

Flowers and leaves of *Cassia fistula* were taken, air-dried and ground by using a blender. 80 grams of ground flowers and ground leaves were weighed using 2 digit balance and then extracted with 320 ml of hexane and ethanol. The extraction was performed at room temperature for 5 days. After that, the solution was filtered using glass cones and filter paper. The filtered solution was evaporated using a rotary evaporator to obtain crude extracts. Then, the crude extracts were weighed and kept for preliminary screening of phytochemicals, antioxidant and antibacterial activities.

Phytochemical screening

Preliminary screening of phytochemicals of crude extracts by ethanol and hexane of *Cassia fistula* was performed and classified by 10 secondary metabolites: alkaloids, flavonoids, anthraquinones, coumarin, saponin, tannin, phlobatannins, terpenoids, steroids and cardiac glycosides (Sreeprasert, 2016) by using color development or sedimentation.

Gas chromatography-mass spectroscopy analysis

Gas chromatography-mass spectroscopy (GC-MS) analysis of dried and fresh leaves e of Indian Laburnum extracted by ethanol was performed using Perkin Elmer Glarus 680 GC equipped with a capillary column made of Elite-5MS. Helium was the carrier gas at a flow rate of 1ml/min. The temperature was

programmed as follows: 50 ºC at 5 ºC/min to 100 ºC and 3 ºC/min to 250 ºC. Identification of phytochemical components was compared with the database of National Institute Standard and Technology MS library (NIST-MS library).

Sample preparation for protein analysis

Fresh and dried leaves of *Cassia fistula* were mashed. A gram of mashed leaves (both of fresh and dried) was weighed and filled with 3 ml of phosphate buffer. Then, it was centrifuged for 60 min. The supernatant was taken and filled in a test tube. 200 µl of sample were mixed with 200 µl of 2X-buffer-treatment and then boiled for 3 min. Finally, it was kept for analyzing protein form.

Study on protein form by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis, SDS-PAGE

Glass plates of the electrophoresis panel were set and then the mixture of acrylamide solution was prepared to obtain 10% concentration of separating gel (H_2O 4.85 ml, 30% acrylamide Mix 3.35 ml, 1.5 M Tris (pH 8.8) 1.65 ml, 10% SDS 100 µl, 10% APS 50 µl, TEMED 3.5 µl).

Acrylamide solution was poured into the space between the glass plates of the electrophoresis set, leaving about 1 cm of the stacking Gel left. Distilled water was added to the gel to prevent polymerization. Then, the gel was allowed to solidify for about 30 min. Next, distilled water was discarded and it was washed with distilled water for 3-4 times. Tissue paper was used to absorb the excess water. The 4% of stacking gel mixture was prepared, H_2O 3.05 ml, 30% acrylamide Mix 670 µl, 1.5 M Tris (pH 6.8) 1.25 ml, 10%SDS 50 µl, 10%APS 60 µl, TEMED 5 µl. A comb was placed in the space between classes to a sample cavity. The stacking gel was poured over the separating gel and left to harden for about 15 min. Then, it was inserted into the electrophoresis set. An electrode buffer solution was added to cover sample cavity and the comb was pulled out. The sample, which mixed with sample buffer and was boiled for 5 min, was filled in the sample cavity at 10 µl per cavity. An electric current of 120 volts was supplied until the color of Bromophenol Blue moved to the bottom of the gel. The gel was slowly removed from the glass and then dyed with Coomassie Brilliant Blue R-250 for 2 h, then it was washed with the Destaining solutions I and II until color bands of the protein was clearly seen and the gel was quite clear.

Antibacterial activity testing by Disc diffusion method

Staphylococcus aureus ATCC 6538 and *Pseudomonas aeruginosa* ATCC 27853, which are generally used in antibacterial activity testing, were placed in a dish containing nutrient agar (NA) and incubated at 37°C for 24 h. Then, two bacterial species were diluted to OD_{600} = 0.01. Next, it was swabbed on the culture medium. A 6 mm paper disc was then placed on the surface of the medium. Tested extracts were pipetted on a paper disc (10 µl/sample). The negative control was the solvents used to dissolve the extracts. The first treatment group was pasteurized with distilled water and the second was performed using 95% ethanol with antibiotic. Chloramphenicol was applied as positive control. It was incubated at 37°C for 24 h. Diameter of inhibition zone was measured in millimeter unit and the results were shown in average± standard deviation (Srihongthong, Kaewsontea, Kongchan, Kiatprasert, & Doungsri, 2017)

Lowest concentration to inhibit bacterial growth tested by Broth diffusion assay

The lowest concentration to inhibit growth of *Staphylococcus aureus* ATCC 6538 and *Pseudomonas aeruginosa* ATCC 27853 was performed by broth diffusion assay. Briefly, a stock solution of crude extracts was diluted. 100 µl of diluted extracts with culture of *Staphylococcus aureus* ATCC 6538 and *Pseudomonas aeruginosa* were maintained at 37°C for 24 h. After that, 100 µl of diluted extracts were transferred into 4.8

ml of BHI broth. This made BHI broth having the same number of bacteria in each tube. Next, the tube was incubated at 37 °C for 24 h. Bacterial growth in each tube was monitored. This experiment was performed triplicately. For the control, 95% ethanol was used instead of plant extracts (Rungsriphanurat, Kammasjesadakun, & Chanwiiayanuchit, 2016).

Results

Condition for separating crude extracts from flowers and leaves of *Cassia fistula*

Flowers and leaves of *Cassia fistula* were crudely extracted to test phytochemical and biological activities. In this study, the solvents used for extraction were ethanol and hexane. Flowers and leaves used were in both fresh and dried forms. After extracting and incubating in the solvents for 3 days, it was filtered and evaporated by using evaporator. It was found that the crude extracts obtained from fresh flowers and leaves were higher than those from the dried. Moreover, when comparing the solvent efficiency, it was found that ethanol provided a better extraction efficiency and a higher yield than hexane.

Chemical properties of flowers and leaves of *Cassia fistula*

After phytochemical properties of flowers and leaves of *Cassia fistula* in both fresh and dried forms were studied and evaluated, there were 10 types, comprising alkaloids, flavonoid, anthraquinone, coumarin, saponin, metanin, phlobatannin, turpinoids, steroids, and cardiac glycoside. It was found that solvents affected the extraction and extract properties. When using ethanol as a solvent, it was found that various substances could be extracted and its chemical properties in flowers and leaves were better than those extracted with hexane. Moreover, when comparing fresh and dried forms, we found that the fresh expressed higher properties than the dried. The phytochemicals found were alkaloids, flavonoids, coumarin, metanin, phlobatannin, turpinoids, steroids, and cardiac glycoside (Table 1).

Table 1 Phytochemical properties and extraction solvents

Properties	Ethanol				Hexane			
	Fresh leaves	Dried leaves	Fresh flowers	Dried flowers	Fresh leaves	Dried leaves	Fresh flowers	Dried flowers
Alkaloids	+	+	+	+	+	–	+	+
Flavonoid	+	+	+	+	+	–	+	+
Anthraquinone	–	–	–	–	–	–	–	–
Coumarin	+	+	+	+	–	–	+	+
Saponin	–	–	–	–	–	–	–	–
Metanin	+	+	–	+	–	–	–	–
Phlobatannin	+	–	–	–	–	–	–	–
Turpinoids	+	+	+	–	–	–	+	+
Steroids	+	+	+	+	+	–	+	+
Cardiac glycoside	+	+	+	+	+	–	–	–

Remarks + interaction – no interaction

An appropriate solvent to extract sample from flowers and leaves of *Cassia fistula*

After extraction conditions and the solvents were studied based on the amount of the obtained crude extracts, it was found that the efficacy of ethanol was better than that of hexane (Figures 1 and 2). The amount of crude extracts obtained from fresh flower extracted by ethanol was 3.1 times higher than that of hexane. For dried flowers, fresh leaves and dried leaves, the amount of crude extracts obtained from ethanol extraction was 3.3, 1.6 and 12.8 times higher than that from hexane extraction, respectively.

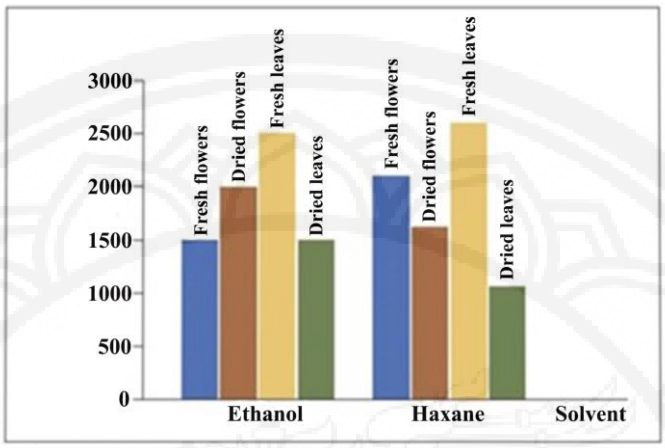

Figure 1 Amount of extraction solvent applying to extract 80 g of flowers and leaves of *Cassia fistula*

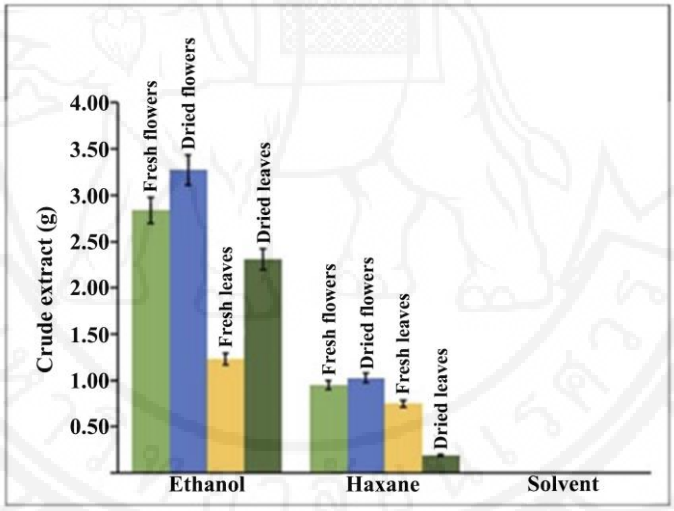

Figure 2 Amount of crude extract from 80 g of flowers and leaves of *Cassia fistula*

Gas chromatography-mass spectrometry analysis (GC-MS)

In this study for GC-MS analysis, we designed to analyze the samples in only fresh and dried leaves because they are found all year round; in contrast, flowers can be blossomed in only summer. The GC-MS analysis of the extract samples revealed the existence of different phytochemical compounds. Based on percentages of peak area, retention time and molecular formula were used to identify phytochemical compounds. Major phytochemical compounds obtained through the analysis of the fresh and dried leaves of *Cassia fistula* are shown in Tables 2 and 3. The results showed that both of (E)-4-(3-Hydroxyprop-1-en-1-yl)-2-methoxyphenyl and thiophene, tetrahydro-2-methyl- Myo-Inositol can be found in both fresh and dried leaves of *Cassia fistula*.

Table 2 Phytochemical components in ethanolic extract of fresh leaves of *Cassia fistula*

S.No	RT	Peak area (%)	Name of the compound	Mw (g/mol)	Molecular formula
1	29.028	2.013	4-O-alpha-D-Glucopyranosyl-D-glucose	360.31	$C_{12}H_{24}O_{12}$
2	30.939	1.194	2-Nonadecanone 2,4-dinitrophenylhydrazine	462.6	$C_{25}H_{42}N_4O_4$
3	31.377	1.486	9-Octadecenoic acid, (2-phenyl-1,3-dioxolan-4-yl)methyl ester, cis-	442.6	$C_{28}H_{42}O_4$
4	32.526	1.867	(E)-4-(3-Hydroxyprop-1-en-1-yl)-2-methoxyphenol	180.2	$C_{10}H_{12}O_3$
5	33.464	1.788	Cyclohexane	84.16	C_6H_{12}
6	34.725	1.994	3-O-Methyl-d-glucose	194.18	$C_7H_{14}O_6$
7	35.300	1.435	Glucitol, 6-O-octyl-	294.38	$C_{14}H_{30}O_6$
8	35.436	2.196	(2S,2′S)-2,2′-Bis[1,4,7,10,13-pentaoxacyclopentadecane]	438.51	$C_{20}H_{38}O_{10}$
9	35.578	1.501	1,3-Dioxolane, 4-methyl-2-pentadecyl-	298.5	$C_{19}H_{38}O_2$
10	35.921	4.973	Neophytadiene	278.5	$C_{20}H_{38}$
11	36.124	2.979	3-Methylmannoside	194.18	$C_7H_{14}O_6$
12	36.231	1.595	Octaethylene glycol monododecyl ether	538.75	$C_{28}H_{58}O_9$
13	36.394	1.887	2H-Pyran-2-acetic acid, tetrahydro-	144.168	$C_7H_{12}O$
14	36.481	1.488	Thiophene, tetrahydro-2-methyl- Myo-Inositol	102.2	$C_5H_{10}S$
15	36.566	1.824	1,4-Dioxan-2-yl hydroperoxide	120.104	$C_4H_8O_4$
16	36.752	3.296	N,N′-Bis(3-aminopropyl)ethylenediamine	174.29	$C_8H_{22}N_4$
17	36.864	6.952	N,N′-Diacetylethylenediamine	144.17	$C_6H_{12}N_2O_2$
18	37.310	1.297	Z,Z-4,16-Octadecadien-1-ol acetate	308.5	$C_{20}H_{36}O_2$

Table 3 Phytochemical components in ethanolic extract of dried leaves of *Cassia fistula*

S.No	RT	Peak area (%)	Name of the compound	Mw (g/mol)	Molecular formula
1	27.629	4.187	Methyl 6-O-[1-methylpropyl]-á -d-galactopyranoside	250.29	$C_{11}H_{22}O_6$
2	32.390	1.337	(E)-4-(3-Hydroxyprop-1-en-1-yl)-2-methoxyphenol	180.20	$C_{10}H_{12}O_3$
3	33.291	0.980	tert-Hexadecanethiol	258.511	$C_{16}H_{34}S$
4	34.482	1.340	Hexa-t-butylselenatrisiletane	505.9	$C_{24}H_{54}SeSi_3$
5	34.925	4.756	1,6-Anhydro-2,3-dideoxy-b-D-threo-hex-2-enopyranose	128.13	$C_6H_8O_3$
6	34.973	1.735	1,2,4-Trioxolane-2-octanoic acid, 5-octyl-, methyl ester	344.486	$C_{19}H_{36}O_5$
7	35.148	1.219	Propane, 1-(1-methylethoxy)-	102.17	$C_6H_{14}O$
8	35.228	1.206	2,3-Di-O-methyl-D-xylopyranose	178.18	$C_7H_{14}O_5$
9	35.771	4.439	13,16-Octadecadienoic acid, methyl ester	294.5	$C_{19}H_{34}O_2$
10	35.981	2.937	Thiophene, tetrahydro-2-methyl- Myo-Inositol	102.198	$C_5H_{10}S$
12	36.174	3.132	1,4-Dioxanyl hydroperoxide	120.1	$C_4H_8O_4$
13	36.209	1.356	alpha-D-Galactopyranose, 6-O-(trimethylsilyl)-, cyclic 1,2:3,4-bis(butylboronate)	384.2	$C_{17}H_{34}B_2O_6Si$
14	36.259	1.940	Ethanol, 2-(1-methylethoxy)-, acetate	146.18	$C_7H_{14}O_3$
15	36.334	5.602	3-Ethylthio-1-propene	102.198	$C_5H_{10}S$
16	36.576	3.194	Heptanoic acid, docosyl ester	298.5	$C_{19}H_{38}O_2$

The amount and pattern of protein found in leaves of *Cassia fistula*

Because fresh leaf extraction gave various chemicals and higher amount of crude extracts than the flower extraction, it was thus applied to study the protein amount for further use in aquaculture. It was found that the protein amount of fresh leaves (28.25 ± 2.32 mg/ml) was 7 times higher than that of dried leaves (4.01 ± 0.9 mg/ml) (Figure 3). On the other hand, in this study of protein, we designed to test the samples in only leaves because they can be found all year round but flowers can be blossomed in only summer. Then, the use of leaves was better than flowers in applicable ways.

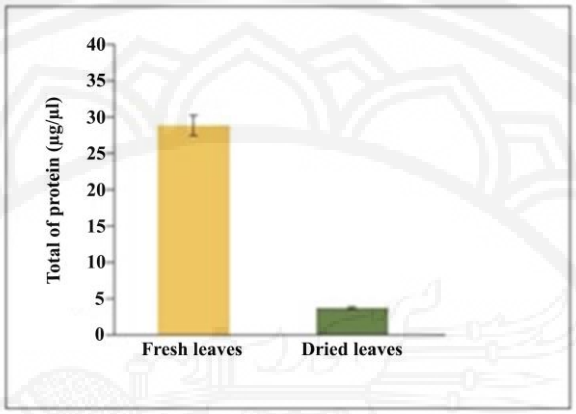

Figure 3 Amount of protein separated and extracted from fresh and dried leaves of *Cassia fistula*

Besides, the forms of protein found in fresh and dried leaves of *Cassia fistula* was studied by using gel electrophoresis technique, it was found that there were 6 important protein bans in fresh leaves: 80, 52, 48, 35 and 23 kDa, while there were only two bands in dried leaves; 71 and 35 kDa (Figure 4).

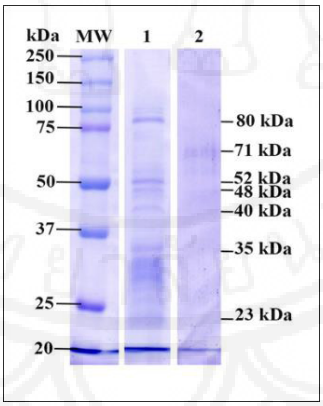

Figure 4 10% SDS-PAGE illustrating forms of protein being separated from fresh and from dried leaves of *Cassia fistula;* where, MW is Standard protein (Bio Rad, company)

Lane 1 protein being separated from fresh leaf of *Cassia fistula* (15 µg/µl)

Lane 2 protein being separated from dried leaf of *Cassia fistula* (3 µg/µl)

The study of bacterial inhibitory capability

For the study of bacterial inhibitory capability, we designed to investigate only in fresh and dried leaves as same as the studies of GC-MS and protein because they can be found all year round. Leaves of *Cassia fistula* were easily extracted by ethanol and obtained various phytochemicals and high amount of protein. Thus, the

were applied to study the bacterial inhibitory capability of *Staphylococcus aureus* and *Pseudomonas aeruginosa* by using two techniques: disc diffusion and dilution methods. We found that the crude extracts from both fresh and dried leaves of *Cassia fistula* could inhibit bacteria, *Staphylococcus aureus*, with its effectiveness slightly different from antibiotics. The inhibition zone measured was 13.7 mm. At the highest crude extract concentration (3 mg/ml), the inhibition zone was 12.67 ± 3.33 mm which was higher efficacy compared to the concentration of 1.5 mg/ ml having inhibit zone of 10.67 ± 3.72 mm. For the concentration of crude extract in between 0.188-0.75 mg/ml, the inhibition zones measured were ranged from 7.33 ± 3.01 to 7.83 ± 3.19 mm. Moreover, the study on the bacterial inhibitory capability by dilution method, it was found that fresh and dried leaves in all concentration levels could inhibit growth of *Staphylococcus aureus* (Table 4).

Table 4 Bacterial inhibitory capability to *Staphylococcus aureus* of fresh and dried leaves of *Cassia fistula*

Treatment condition of *Staphylococcus aureus*	Plant part	disc diffusion inhibition zone	dilution method
negative control	Fresh leaves	-	-
positive control		13.7 ± 0.14 (++)	-
antibiotic (cholramphenicol)			
concentration of 3 mg/ml		12.67 ± 3.33 (++)	-
concentration of 1.5 mg/ml		10.67 ± 3.72 (+)	-
concentration of 0.75 mg/ml		9.17 ± 3.92 (+)	-
concentration of 0.375 mg/ml		7.83 ± 3.19 (+)	-
concentration of 0.188 mg/ml		7.33 ± 3.01 (+)	-
negative control	Dried leaves	-	-
positive control		13.7 ± 0.14 (++)	-
antibiotic (cholramphenicol)			
concentration of 3 mg/ml		12.00 ± 3.41 (++)	-
concentration of 1.5 mg/ml		10.33 ± 3.08 (+)	-
concentration of 0.75 mg/ml		9.33 ± 2.25 (+)	-
concentration of 0.375 mg/ml		8.17 ± 2.04 (+)	-
concentration of 0.188 mg/ml		7.17 ± 1.83 (+)	-

Remark 1 Concentration of crude extract used was 3 mg/ml

Inhibition zone found as -

No Inhibition zone found in 9-11 mm as +, 12-15 mm as ++, 16-19 mm as +++, 20-23 mm as ++++, more than 23 mm as +++++ (Wang-Amnauyporn & Saiprapong, 2007)

Remark 2 Bacterial inhibitory capability studied by dilution method

+ cannot inhibit bacterial growth due to turbidity

- can inhibit bacterial growth and found dead bacteria at the tube bottom and culture media being clear

Bacterial inhibitory capability to *Pseudomonas aeruginosa*

After bacterial inhibitory capability of crude extract to *Staphylococcus aureus* was studied, it was used to study the ability to inhibit the growth of *Pseudomonas aeruginosa* by using two techniques: disc diffusion method and dilution method, as in previous studies. We found that both fresh and dried leaves of *Cassia fistula* could inhibit growth of *Pseudomonas aeruginosa* bacteria. The effectiveness was slightly different from antibiotics. The inhibition zone was 13.5 mm. At the highest crude extract concentration of 3 mg/ml, the inhibition zone was 12.17± 3.92mm which was better than that concentration of 1.5 mg/ml with inhibition

zone of 11.17± 3.87 mm. For the concentration levels of crude extract in between 0.188-0.75 mg/ml, the inhibition zones measured were ranged from 7.83 ± 2.79 to 8.67± 3.33 mm. Moreover, the study on the bacterial inhibitory capability by dilution method, it was found that fresh and dried leaves in all concentration levels could inhibit growth of *Pseudomonas aeruginosa* (Table 5).

Table 5 Bacterial inhibitory capability to *Pseudomonas aeruginosa*

Treatment condition of *Pseudomonas aeruginosa*	Plant part	disc diffusion inhibit zone	dilution method
negative control	Fresh leaf	-	-
positive control antibiotic (cholramphenicol)		13.5 ± 0.08 (++)	-
concentration of 3 mg/ml		12.17± 3.92 (++)	-
concentration of 1.5 mg/ml		11.17± 3.87 (+)	-
concentration of 0.75 mg/ml		10.00± 3.35 (+)	-
concentration of 0.375 mg/ml		8.67 ± 3.33 (+)	-
concentration of 0.188 mg/ml		7.83 ± 2.79 (+)	-
negative control	Dried leaf	-	-
positive control antibiotic (cholramphenicol)		13.5 ± 0.08 (++)	-
concentration of 3 mg/ml		12.33± 3.88 (++)	-
concentration of 1.5 mg/ml		11.00± 4.43 (+)	-
concentration of 0.75 mg/ml		10.00± 3.74 (+)	-
concentration of 0.375 mg/ml		8.17 ± 3.19 (+)	-
concentration of 0.188 mg/ml		7.33 ± 2.58 (+)	-

Remark 1 Concentration of crude extract used was 3 mg/ml
Inhibition zone found as -
No Inhibition zone found in 9-11 mm as, 12-15 mm as ++, 16-19 mm as +++, 20-23 mm as ++++, more than 23 mm as +++++ (Wang-Amnauyporn & Saiprapong, 2007)

Remark 2 Bacterial inhibitory capability studied by dilution method
+ cannot inhibit bacterial growth due to turbidity
- can inhibit bacterial growth and found dead bacteria at the tube bottom and culture media being clear

Discussion

Plant extraction can be performed by many methods, depending on plant species, substances that need to be extracted or objectives of the study. Initially, the extraction of plant yielded crude extracts that still have a mixture of various chemical compositions, which include both the chemicals having pharmaceutical effects and without that (inert substances) (Kaeouphai, 2013). An important factor in extracting the plant material is choosing the appropriate solvent in order to obtain the substance according to the purpose and get the highest amount of substance. Searching for information about plants or substances in plants that are interesting to study; therefore, is an important starting point for selecting suitable solvents. In general, the solvent must be able to dissolve substances that need to be extracted. The solvent must not react with the substance to be extracted. In the case of color separation, it must be colorless or odorless. Besides, in the case of odor separation, it must be non-toxic, low boiling point and easy to separate from substances that need to be

extracted. It should be not dissolved in a homogeneous substance to extract or dissolve impurities and unwanted substances. Moreover, it should be easily available and inexpensive.

Phytochemical analysis

Cassia fistula is a tropical plant and widely found in Thailand. It is a plant that has medicinal properties (Irshad et al., 2013). Phytochemicals antioxidants that are currently extensively studied are phytochemical substances that are found in plants. It is mainly a secondary product that plants synthesize for certain purposes, such as protecting themselves from germs and insects or expressing color. Examples of phytochemical substances are tannin, polyphenolic compound, anthraquinones, saponins, alkaloids and coumarins. There are many phytochemicals in plants, each with different effects, but most of them are antioxidants due to their complex structure and tendency to give electrons (Irshad et al., 2013; Srihongthong et al., 2017).

When studying phytochemical of flowers and leaves of *Cassia fistula* in both fresh and dried forms by evaluating all 10 types of properties: alkaloids, flavonoid, anthraquinone, coumarin, saponin, metanin, floba tannin, terpenoid, steroid and cardiac glycoside, it was found that solvents affected the extraction and properties of extracts. When using ethanol as a solvent, it is found that various substances could be extracted more than using hexane. Moreover, it was better to apply in testing the chemical properties of the flowers and leaves of *Cassia fistula* than applying hexane as a solvent. When comparing extraction in fresh and dried leaves and fresh and dried flowers, it was found that fresh leaves had the highest properties in various forms of phytochemical, which can be assessed in total of 8 properties: alkaloids, flavonoids, coumarin, metanin, floba tannin, terpenoid, steroid and cardiac glycoside. The results of this study correspond to the study of Jundee (2016) which tested phytochemical having antioxidant and antibacterial activities of crude extracts from roots and wild grape vines extracted by 3 different solvents: 95% methanol, 95% ethanol, and 35% white liquor. They found 4 groups of phytochemicals, including cardiac glycoside, tannin, terpenoid and saponin.

Parag, Vanita, and Bhanu (2014) studied the physicochemical and phytochemical properties of the leaves of *Ampelocissus latifolia* (ROXB.) extracted by using soxhlet with 14 solvents. Many phytochemicals were found: alkaloids, saponins, carbohydrates, glycosides, tannins, flavonoids, steroids, phenols, proteins, monosaccharides, hexose sugars and starch. This difference from this study might be caused by a variety of plants and solvents.

The GC-MS analysis of the extract samples revealed the existence of different phytochemical compounds. Based on percentages of peak area, retention time and molecular formula used for the identity of phytochemical compounds. Major phytochemical compounds obtained through the analysis of the fresh leaves and dried leaves of *Cassia fistula* have been shown that both (E)-4-(3-Hydroxyprop-1-en-1-yl)-2-methoxyphenyl and thiophene, tetrahydro-2-methyl- Myo-Inositol can be found in dried leaves and fresh of *Cassia fistula*. **Both of these compound were also found in ethyl acetate extract of *Tinospora cordifolia* (Jain, Dhuria, Sharma, & Bothra, 2018) and *Cleistanthus collinus* leaf (Suman, Chakkaravarthi, & Elangomathavan, 2013).** The first is rich in phenolic compounds having the properties of anticancer, antimicrobial, antioxidant activity (Jain et al., 2018), whereas thiophene, tetrahydro-2-methyl- Myo-Inositol have the property of antimicrobial activity (Suman et al., 2013). 2-Nonadecanone 2, 4-dinitrophenylhydrazine and neophytadiene were found in fresh leaves of *Cassia fistula* that is used for fragrance (Muthulakshmi, Margret, & Mohan, 2012) and antimicrobial (Hazarika, Goswami, Dutta, & Hazarika, 2002). Moreover, neophytadiene is a good analgesic,

antipyretic, anti-inflammatory, antimicrobial, and the antioxidant compound that was reported by Raman et al (2012).

In the case of dry leaves of Indian Laburnum, the GC-MS analyses found that major phytochemical compounds were composed of Methyl 6-O-[1-methylpropyl]-alpha-d-galactopyranoside, tert-Hexadecanethiol, 13,16-Octadecadienoic acid, methyl ester, and Heptanoic acid, docosyl ester. **The results showed the presence of many phenolic compounds that similar to report of (Swayamsiddha, Meenakshi, Ravichandran, & Brindha, 2014).** These compounds have been reported that its presence has properties of anticancer activity (Swayamsiddha et al., 2014,), Enzyme activators (Rajendran, Bharathidasan, & Sureshkumar, 2017) and antimicrobial activity (Abubakar & Majinda, 2016).

The forms of protein of *Cassia fistula* using SDS-PAGE

The SDS-PAGE technique is simple and can be applied in separating proteins according to their molecular weight. Therefore, it is widely used for the characterization of biological objects. In this study, it was applied to separate protein extracted from leaves of *Cassia fistula*. The important protein bands found was in the range of 23 to 80 kDa. There were 7 bands: 80, 71, 52, 48, 40, 35 and 23 kDa, respectively. It was slightly different from the study of Chernyshenko, Chernyshenko, and Musatenko (2016) which studied the protein form in the leaves and roots of *Ophioglossum vulgatum* by using SDS-PAGE technique and found that the protein in both leaves and roots had a molecular weight of 112 ± 1.3 , 57 ± 1.0, 45 ± 1.2, 35.5 ± 0.6, 25.8 ± 0.5, 18 ± 1.5 kDa, respectively. This might be due to different plants, even using the same buffer for extracting. Moreover, this study is also different from the studies of Chittora and Purohit (2012) which isolated and studied the form of protein in seed of *Abrus precatorius L.* and used a buffer for extraction; 200 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM EDTA, 0.1% $MgCl_2$, 15% glycerol, 2.8 mM PMSF and 10 mM mercaptoethanol. This is different from our study because the buffer used in the extract contains 0.1% $MgCl_2$ as an ingredient, which could separate proteins of various sizes, from 20-143 kDa (40 bands).

Bacterial inhibitory capability testing

Cassia fistula is a tropical plant and widely found in Thailand. It is a plant that has medicinal properties (Irshad et al., 2013). Its leaf extract, which is extracted using otro alcohol can inhibit the growth of gram-positive bacteria such as *Staphylococcus aureus* and *Streptococcus spp.*, gram-negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa* and fungi such as *Aspergillus niger*, *Aspergillus clavatus* and *Candida albicans* (Bhalodia & Shukta, 2011). Moreover, Panda et al. (2011) found that extracts from *Cassia fistula* leaf extracted with petroleum ether, chloroform, ethanol, methanol and water could inhibit growth of gram-positive bacteria; *Staphylococcus epidermiglis*, *Bacillus licheniformis* and *Bacillus subtilis*.

From the problem of various pathogenic bacteria, there have been many studies and research that have used extracts to inhibit the different types of pathogenic bacteria in both gram-positive and gram-negative bacteria, with their properties and inhibitory capability varying depending on the type and part of plant. For example, Srihongthong et al. (2017) studied bacteriological inhibition properties of 12 plant extracts: guava leaf, guava stalks, gourd leaf, young mulberry leaf, old mulberry leaf, mulberry fruit, rain tree leaf, betel leaves, centella asiatica, nail-fringed glass, and bamboo grass leaf after extraction with distilled water and 95% ethanol. Then, they were applied to inhibit the growth of 4 strains of bacteria: *Escherichia coli* ATCC 10536, *Listeria monocytogenes*, *Staphylococcus aureus* ATCC 6538 and *Streptococcus pneumoniae* ATCC 49619 using disc diffusion method. It was found that guava leaf, mulberry fruit, betel leaf and asiatica leaf being extracted by

ethanol could inhibit all strains of bacteria tested, with inhibition zone of 8.70-15.70 mm. Extracts of guava leaf extracted by water expressed highest inhibitory capability with an inhibition zone of *Escherichia coli* ATCC 10536 and *Staphylococcus aureus* ATCC 6538 as 23.00 ± 1.00 and 14.70± 0.58 mm, respectively.

Rungsriphanurat et al. (2016) reported that after extraction by using 95% ethanol, the extracts of turmeric, *Chedhed alata*, eight red sandalwood, sandalwood, black pepper, canopy, wild thorn, fennel, *Chebulic Myrobalans* and cinnamon showed inhibitory capability to *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* and *Escherichia coli* ATCC 25922. After testing by agar well diffusion method, it was found that all Thai herbal extracts inhibited *Staphylococcus aureus* ATCC 25923. Seven Thai herbal extracts inhibited *B.cereus*. Five Thai herbal extracts inhibited all species of bacteria tested. The extracts of Sappan tree showed highest inhibitory capability to three species of bacteria. After minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) was tested by broth dilution method, they found that Sappan tree extracts had MIC equal to MBC to *Staphylococcus aureus* ATCC 25923, *B. cereus* and *E. coli.* ATCC 25922 as 8, 2 and 1 mg/ml, respectively.

In this study, the antibacterial activity was tested by disc diffusion method and dilution method. It was found that all crude extracts inhibited gram-positive bacteria, *Staphylococcus aurenas* and gram-negative bacteria, *Pseudomonas aeruginosa* compared to antibiotics, chloramphenicol. By using crude ethanol extracted from fresh leaf, it gave the highest inhibitory effect to *Staphylococcus aurenas* when compared with other crude extracts. The inhibition zone was 12.67 ± 3.33 mm and the crude ethanol extracted from dried leaf gave the highest inhibitory effect to *Pseudomonas aeruginosa* when compared with the other. The inhibitory zone was 12.33 ± 3.88 mm. In the study of the antibacterial effect test by dilution method, it was found that all crude extracts in all concentration levels could inhibit *Staphylococcus aurenas* and *Pseudomonas aeruginosa*.

Our study is different from the study of Nithikulworawong (2012) which studied the efficacy of extracts from Sirindhorn Wanli herbs to inhibit *Streptococcus agalactiae* in Nile tilapia using the extracts of flowers and leaves extracted by 95% acetone and ethanol. It was found that the extracts using 95% ethanol had the highest inhibitory capability to *Streptococcus agalactiae* with inhibition zone of 15.53 ± 0.65 mm. Based on all results, it showed that the extract could be used to study bacterial inhibition by both disc diffusion and dilution method.

The results of the phytochemical and biological activities of *Cassia fistula* extracts showed that if conducting a more detailed study, *Cassia fistula* is an alternative plant that has good biological activity and interesting phytochemicals that can be further applied.

Conclusion and Suggestions

After the extracts of *Cassia fistula* by using ethanol and hexane as extraction solvents were studied for their phytochemical by color development and precipitation testing, the protein form by using SDS-PAGE technique (10%), bactericidal inhibitory capability by disc diffusion method and dilution methods, the results found could be summarized as follows:

1. After studying phytochemical of flowers and leaves of *Cassia fistula* in both fresh and dried forms by evaluating all 10 properties, we found that fresh leaves have more properties (8 types); alkaloids, flavonoids, kumarin, metanins, phlobatannins, terpenoid, steroids and cardiac glycosides.

2. The GC-MS analysis of fresh and dried leaves of *Cassia fistula* showed that both of (E)-4-(3-Hydroxyprop-1-en-1-yl)-2-methoxyphenyl and thiophene, tetrahydro-2-methyl- Myo-Inositol.

3. After studying protein forms of *Cassia fistula* leaves by using SDS-PAGE technique, we found important protein bands in the range of 23 to 80 kDa. There were 7 bands: 80, 71, 52, 48, 40, 35 and 23 kDa, respectively.

4. After studying the bacterial inhibitory capability by using disc diffusion and dilution methods, it was found that the crude extracts had inhibitory activity on *Staphylococus aurenas* and *Pseudomonas aeruginosa* in comparison with antibiotics (chloramphenicol). The crude extracts from fresh leaves could inhibit *Staphylococus aureus* better than from the dried. At the concentration of 3 mg/ml, the maximum inhibit zone was 12.67 ± 3.33 mm. In contrast, the results of an inhibition study in gram-negative bacterial, *Pseudomonas aeruginosa*, showed that the extract from dried leaves (3 mg/ml) gave the highest inhibition zone as 12.33 ± 3.88 mm. In the study of bacterial inhibitory capability by dilution method, it was found that all crude extracts concentration could inhibit *Staphylococus aurenas* and *Pseudomonas aeruginosa*. Even though this study tends to be success in a crude extraction, there should be other experiments emphasizing on purification and dried extraction methods for more beneficial applications in the future.

Acknowledgments

The authors would like to thank Dr.Saowaluck Srihongthong and Dr.Paweena Saleethong for comments on an earlier draft of this manuscript.

References

Abubakar, M. N., & Majinda, R. R. T. (2016). GC-MS Analysis and Preliminary Antimicrobial Activity of *Albizia adianthifolia* (Schumach) and *Pterocarpus angolensis* (DC). *Medicines*, *3*(3), 1-9.

Bhalodia, N. R., & Shukla, V. J. (2011). Antibacterial and antifungal activities from leaf extracts of *Cassia fistula* l.: An ethnomedicinal plant. *Journal of Advanced Pharmaceutical Technology & Research, 2,* 104-109.

Chanwun, T., Rongsawat, P., U-taynapun, K., & Chirapongsatonkul, N. (2016). Effect of *Cassia fistula* leaf Extract on Growth of a Rubber Plant Black Strip Disease Pathogen *Phytophthora palmivora*. *Songklanakarin Journal of Plant Sci*ence, *3*(1), 66-71.

Chernyshenko, M. S., Chernyshenko, V. O., & Musatenko, L. L. (2015). SDS-PAGE *of Ophioglossum vulgatum* Proteins. *International Journal of Physical and Social Sciences, 9*(1), 1-7.

Chitmanat, C. (2013). Effects of herbal products on fish immunity. *KKU Research Journal, 18*(2), 257-269.

Chittora, M., & Purohit, S. D. (2012). Optimization of SDS-PAGE conditions and analysis of seed protein diversity in *Abrus precatorius* genotypes with different seed coat colour. *International Journal of Life Sciences Biotechnology and Pharma Sciences, 1*(2), 269-277.

Irshad, M., Ahmad, A., Zafaryab, M., Ahmad, F., Manzoor, N., Singh, M., & Rizvi, M. M. (2013). Composition of Cassia fistula oil and its antifungal activity by disrupting ergosterol biosynthesis. *Nat Prod Commun*, *8,* 261-264.

Jain, D., Dhuria, R. K., Sharma, T., & Bothra, T. (2018). Phytochemical analysis of ethyl acetate extract of *Tinospora cordifolia* by mass spectrometry. *International Journal of Chemical Studies, 6*(2), 2336-2340.

Hazarika, S., Goswami, N., Dutta, N. N., & Hazarika, A. (2002). Ethyl oleate synthesis by porcine pancreatic lipase in organic solvent. *Chemical Engineering Journal*, *85,* 61-68.

Jundee, C. (2016). *Antibacterial activities of wild grape (Ampelocissus amrtiniplanch) extract.* Department of Science, Burapha University.

Kaeouphai, K. (2013). *Antioxidant activity, total phenolic and chemical constituents of the costus speciosus smith, crude extract from Siridhorn peat swamp forest, Narathiwat.* Faculty of Science, Burapha University.

Muthulakshmi, A., Margret, R. J., & Mohan, V. R. (2012). GC-MS Analysis of Bioactive Components of *Feronia elephantum* Correa (Rutaceae). *Journal of Applied Pharmaceutical Science, 2*(2), 69-74.

Nithikulworawong, N. (2012). Efficacy of Bauhinia sirindhorniae on Resistance to Against *Streptococcus agalactiae* in Nile tilapia (*Oreochromis niloticus*). *KKU Research Journal, 17*(5), 715-724.

Panda, S. K., Sujogya, K., & Mohanty, G. (2011). Antibacterial activities and phytochemical analysis of *Cassia fistula* (Linn.) leaf. *Journal of Advanced Pharmaceutical Technology & Research, 2,* 62-67.

Parag, P. A., Vanita, K., & Bhanu, R. (2014). Physico Chemical and Phytochemical Evaluation of *Ampelocissus latifolia* (Roxb.) Planch. Leaves. *International Journal of Life Sciences Biotechnology and Pharma Sciences*, *6*(6), 504-507.

Rajendran, P., Bharathidasan, R., & Sureshkumar, K. (2017). GC-MS Analysis of Phyto-Components in Raw and Treated Sugarcane Juice. *International Journal of Current Microbiology and Applied Sciences*, *6*(7), 51-61.

Raman, B. V., Samuel, L. A., Saradhi, M. P., Rao, B. N., Krishna, A. N. V., & Radhakrishnan, T. M., (2012). Antibacterial, antioxidant activity and GC-MS analysis of *Eupatorium odoratum*. *Asian Journal of Pharmaceutical and Clinical Research, 5*(2), 99-106.

Rungsriphanurat, W., Kammasjesadakun, P., & Chanwiiayanuchit, I. (2016). Antibacterial Activities of Ten Thai Herbal Extracts against *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* and *Escherichia coli* ATCC 25922. *Huachiew Chalermprakiet Sci Technol J*, *19*(38), 35-48.

Sreeprasert, J. (2016). *Phytochemical screening and biological activities of Dolichandrone serrulata. Master degree.* Faculty of Science, Burapha University.

Srihongthong, S., Kaewsontea, P., Kongchan, J., Kiatprasert, P., & Doungsri, K. (2017). The Screening of plant extracts for determining antibacterial activity, In *Proceeding of 8th Innovation and Technology conferences* (pp. 253-261). Rajamangala University of Technology Isan Surin Campus: Surin.

Swayamsiddha, P., Meenakshi, S. M., Ravichandran, N., & Brindha, P., (2014). Anticancer activity of ethaolic extract of Solanum torvum SW. *International Journal of Current Microbiology and Applied Sciences, 6*(1), 93-98.

Suman, T., Chakkaravarthi, K., & Elangomathavan, R. (2013). Phyto-chemical profiling of *Cleistanthus collinus* leaf extracts using GC-MS analysis. *Research Journal of Pharmacy and Technology*, *6*(11), 1173-1177.

Tebsun, K., Kronkpong, L., Rodloy, A., & Jintasataporn, O. (2016). *The Effect of Turmeric and Kariyat Crude Extracts on Immunity and Growth Performance of Fancy Carp (Cyprinus carpio Linnaeus, 1758).* Thailand: Inland Fisheries Research and Development Division, Department of Fisheries, Ministry of Agriculture and Cooperatives.

Wang-Amnauyporn, T., & Saiprapong, R. (2007). *Antimicrobial and antioxidant activity of some Thai medical plants.* Bangkok: University of the Thai chamber of commerce.