Original Article

Cellular antioxidant activity of Samanachan remedy and its plant ingredients

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Abstract

Introduction: Samannachan remedy is used to modulate human body elements in cancer patients at Khampramong temple in Sakhon Nakhon province. Samanachan remedy consists of 10 herbal plants namely Kam-lang-sue-krong *(Betula alnoides)*, Maa-kra-tuep-rong *(Ficus foveolata)*, Chang-naw *(Ochna integerrima)*, Kra-jon-nao *(Coptosapelta flavescens)*, Tra-kri-ton *(Litsea cubeba)*, Khan-thong-pha-ya-bath *(Suregada multiflora)*, Ya-nang-dang *(Bauhinia strychnifolia)*, Faang *(Caesalpinia sappan)*, Fa-tha-lai-jone *(Andrographis paniculata)* and Ham *(Coscinium fenestratum)*.

Methods: The extracts of Samanachan remedy and its plant ingredients were prepared by maceration and decoction. All extracts were investigated for the inhibition of superoxide formation measured by NBT reduction using human promyelocytic leukemia cell line (HL-60).

Results: The ethanolic and aqueous extracts of Samanachan remedy inhibited superoxide formation with IC_{50} 31.45 ± 4.92 µg/ml and IC_{50} 47.18 ± 4.74 µg/ml, respectively. The ethanolic extract of *C. sappan* showed the highest activity (IC_{50} 5.75 ± 0.86 µg/ml) when compared with other extracts.

Conclusions: The ethanolic and aqueous extract of Samannachan remedy showed moderate antioxidant activity as compared to the positive control.

Keywords: Samanachan remedy, Antioxidant, Khampramong temple, Thai traditional remedy, Cancer, Superoxide inhibition

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Introduction

Free radicals are highly reactive chemicals that created when an atom or a group of atoms either gains or loses an electron. Free radicals are formed naturally in the body and play an important role in many normal cellular processes. However, free radicals can be hazardous to the body and damage all major components of cells, including DNA, proteins, and cell membranes. The damage to cells caused by free radicals, especially the damage to DNA, may be responsible for the development of cancer and other health conditions.[1, 2] Antioxidants oppose the effects of free radicals and protect cells from damage.[3] Natural antioxidants are contained in many medicinal plants which reduce excessive free radicals and degenerative diseases, especially cancer.[4]

Cancer was the second leading cause of death, with 9.6 million deaths globally in 2018.[5] In Thailand, cancer is the leading cause of death for consecutive years and is increasing in every year. There are many treatments for cancer today including chemotherapy, surgery, radiation therapy, immunotherapy.[6] In addition, herbal treatment is one of the alternative treatments for cancer. Arokhayasala Khampramong temple offers alternative treatments for cancer patients. The aim of Arokhayasala is not merely to cure disease but also to understand and provide for the needs of patients' lives. Even towards the end of the patient's life there is preparation for the patient to leave this world peacefully. This temple serves cancer patients in alternative medicine including natural therapies, herbs, prayers, meditation, art therapy, music therapy, yoga. This is holistic care and healing of the whole being of the patient: physically, mentally and spiritually, along with taking herbal medicine for cancer.[7]

Samanachan (SMC) is one of the remedies that was developed by Paponpatchara the abbot of Khampramong temple who founded Arokhayasala, Khampramong temple, Sakhon Nakhon province. This remedy used for cancer patients in Arokhayasala where it is used as an adaptogenic drug.[8] For administration, Samanachan remedy is boiled with water and given to cancer patients twice daily (60ml/each) for 2 weeks then, cancer patients also receive Ya-yod-kae-ma-reng remedy to treat cancer. Samanachan remedy consists of 10 herbal plants in varying proportions namely Kam-lang-sue-krong *(Betula alnoides)*, Maa-kra-tuep-rong *(Ficus foveolata)*, Chang-naw *(Ochna integerrima)*, Kra-jon-nao *(Coptosapelta flavescens)*, Tra-kri-ton *(Litsea cubeba)*, Khan-thong-pha-ya-bath *(Suregada multiflora)*, Ya-nang-dang *(Bauhinia strychnifolia)*, Faang *(Caesalpinia sappan)*, Fa-tha-lai-jone *(Andrographis paniculata)* and Ham *(Coscinium fenestratum)*.

The ethanolic extract of Samanachan remedy has been reported for cytotoxic activity against HepG2 and KKU-M156 cells with IC_{50} values 41.74 ± 6.87 and 33.17 ± 1.17 µg/ml, respectively.[9] On the other hand, there has been no report on antioxidant activitiy. Consequently, this study aimed to investigate the antioxidant activitiy of Samanachan extracts and its plant ingredients. The results of this research are likely to support ongoing use of this remedy for cancer treatment.

Methods

Extract preparation

Ingredients plant of Samanachan remedy were collected in Kanchanaburi province and all plants were identified by comparison with authentic voucher specimens that were kept in the herbarium of Southern Center of Thai Medicinal Plants, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand. The voucher specimen was shown in Table 1. All plant material was dried at 50 °C, and ground into powder. They were then weighed and mixed to be Samanachan remedy in the proportion of the recipe of Samanachan remedy at Khampramong temple. Samanachan remedy extracts were obtained by two methods, including maceration and decoction. Samanachan remedy and its ingredients were

macerated with 95% ethanol for 3 days and repeated twice times (total 3X) with the residue. The extracts were dried using a rotary evaporator. The aqueous extracts of Samanachan remedy and its ingredients were boiled in distilled water for 15 minutes and filtered. This procedure was repeated twice times (total 3X) on the residue. Next, they were filtered using Whatman NO.1 filter paper and concentrated to dryness by lyophilizer. Percentage yields was calculated by the formula below.

$$\%\text{Yield} = \frac{\text{Weight of extract(g)x100}}{\text{Weight of dried powder(g)}}$$

Table 1 List of plant materials in Samanachan remedy

Scientific Name	Family Name	Thai name	Part used	Code	Specimen voucher
Betula alnoides Buch. Ham.	BETULACEAE	Kam lang sue krong	Bark	BA	SKP 024020101
Ficus foveolata Wall.	MORACEAE	Maa kra tuep rong	Stem	FF	SKP 117060601
Ochna integerrima (Lour.) Merr.	OCHNACEAE	Chang naw	Stem	OI	SKP 218150901
Coptosapelta flavescens Korth.	RUBIACEAE	Kra jon nao	Stem	CFl	SKP 165030601
Litsea cubeba (Lour).	LAURACEAE	Tra kri ton	Stem	LC	SKP 096120301
Suregada multiflorum (A.Juss) Baill.	EUPHORBIACEAE	Khan thong pha ya bath	Stem	SM	SKP 071191301
Bauhinia strychnifolia Craib.	LEGUMINOSAE (FABACEAE)	Ya nang dang	All parts	BS	SKP 098021901
Caesalpinia sappan Linn.	CAESALPINIODEAE	Faang	Stem	CS	SKP 072031901
Andrographis paniculata (Burm.f.) Wall. Ex Nees	ACANTHACEAE	Fa tha lai jone	All parts	AP	SKP 001011601
Coscinium fenestratum (Gaertn.) Colebr.	MENISPERMACEAE	Ham	Stem	CFe	SKP 114030601

Antioxidant activity

Nitroblue tetrazolium (NBT) dye reduction assay[10]

Human cell lines

Human promyelocytic leukemia cells (HL-60) were cultured in RPMI 1640 supplemented with 10% heated fetal bovine serum, 50 IU/ml penicillin and 50 µg/ml streptomycin. The cells were maintained at 37 °C in an incubator with 5% CO_2 and 95% humidity. To induce myeloid differentiation, HL-60 cells were cultured for 6 days in RPMI 1640 containing 1.3% DMSO to differentiate into mature cells.

Preparation of sample solution

Ethanolic extracts were mixed in sterile dimethyl sulfoxide (DMSO) and the aqueous extracts were mixed in sterile water and then filtered through 0.22 µm filter. The extracts were diluted in medium to produce required concentrations. A hundred microliters of each concentration were added to each centrifuge tube to obtain final concentrations of 0.01 - 100 µg/ml.

Procedure of inhibitory effect by Nitroblue tetrazolium (NBT) dye reduction assay

Intracellular superoxide formation was quantified by nitroblue tetrazolium reduction assay (NBT). Differentiated HL-60 1 x 10^6 cells were incubated with various dilutions of the extract dissolved in Hanks' balanced salt solution (HBSS) 500 µl at 37 °C in an incubator with 5% CO_2 and 95% humidity for 15 minutes. Further incubation with 500 ng/ml phorbol 12-myristate 13-acetate (PMA) and 1.25 mg/ml nitroblue tetrazolium (NBT) solution was performed for another hour, then, 2 ml of 1N HCl was added. After vortexing and centrifugation at 4,000 rpm for 10 minutes, the supernatant was removed and cells dissolved in 300 ml DMSO. Then, 100 µl of sample solution was added into 96 well plates. The absorbance was measured at 572 nm using a microplate reader. The inhibition of each concentration of the extract against superoxide formation measured by NBT reduction was calculated by the following equation.

$$\% \text{ NBT reduction} = \frac{(\text{Control O.D.} - \text{Sample O.D.})}{\text{Control O.D.}} \times 100$$

Chemical fingerprint of Samanachan extract

The chemical fingerprint study was performed using high performance liquid chromatography (HPLC) system, with ultraviolet visible (UV-vis) detector (Spectromonitor® 4100) and automatic injector (Spectra System AS3500). The reversed-phase column was ZORBAX Eclipse XDB-C18 Analytical 4.6 x 250 mm. 5 - micron Agilent. The mobile phase consisted of 0.1% phosphoric acid and acetonitrile. The flow rate was 1 ml/min with UV absorbance detection at 254.8 nm. The operating temperature was maintained at room temperature. 10 milligrams ethanolic extract of Samanachan remedy was dissolved in 1 ml methanol, and then sonicated. A stock solution of brazilin was prepared at a concentration of 1.0 mg/ml with DMSO.

Results

The yields of ethanolic extract and aqueous extract of SMC were 4.77% and 9.51%, respectively. For the ethanolic extract of SMC's plant ingredients, *Litsea cubeba* (LC) extract showed the highest percentage yield with 9.45%. The aqueous extract of *Coptosapelta flavescens* (CLF) showed the highest percentage yield (18.16%), as shown in Table 2.

Table 2 The yield (%) of Samannachan remedy and its plant ingredients

Sample	Extraction	%Yield
Samanachan remedy	Ethanolic	4.77
	Aqueous	9.51
Betula alnoides Buch. Ham.	Ethanolic	4.74
	Aqueous	9.98
Ficus foveolate Wall.	Ethanolic	2.59
	Aqueous	10.26
Ochna integerrima (Lour.) Merr.	Ethanolic	3.42
	Aqueous	8.79
Coptosapelta flavescens Korth.	Ethanolic	5.94
	Aqueous	18.16
Lissea cubeba Pers.	Ethanolic	9.45
	Aqueous	3.86
Suregada multiflorum (A.Juss) Baill.	Ethanolic	3.65
	Aqueous	3.54
Bauhinia strychnifolia Craib.	Ethanolic	3.18
	Aqueous	8.00
Caesalpinia sappan Linn.	Ethanolic	6.24
	Aqueous	7.63
Andrographis paniculata	Ethanolic	4.19
(Burm.f.) Wall. Ex Ness.	Aqueous	16.70
Coscinium fenestratum	Ethanolic	4.81
(Gaertn.) Colebr.	Aqueous	11.64

The results of nitroblue tetrazolium (NBT) dye reduction assay of Samanachan remedy and its plant ingredients are shown in Table 3 and Figure 1. The results showed that the ethanolic extract of Samanachan remedy exhibited stronger antioxidant activity than the aqueous extract with EC_{50} values of 31.45 ± 4.92 and 47.18 ± 4.74 µg/ml, respectively. However, Samanachan extract still showed lower activity than propyl gallate as a positive control (9.46 ± 2.52 µg/ml). For plant ingredients, the ethanolic extract of *Caesalpinia sappan* showed the highest activity against intracellular superoxide formation with an EC_{50} value of 5.75 ± 0.86 µg/ml which was higher than the positive control.

Table 3 EC_{50} values of Samanachan remedy and its ingredient plant extracts in scavenging PMA-stimulated superoxide production in HL-60 cells measured by the NBT reduction (mean ± SEM), (n = 3)

Scientific name	Extract	EC_{50} (µg/ml)
Betula alnoides Buch. Ham.	Ethanolic	15.24 ± 1.16
	Aqueous	>100
Ficus foveolata Wall.	Ethanolic	43.77 ± 2.18
	Aqueous	>100
Ochna integerrima (Lour.) Merr.	Ethanolic	18.83 ± 4.64
	Aqueous	>100
Coptosapelta flavescens Korth.	Ethanolic	>100
	Aqueous	>100
Litsea cubeba Pers.	Ethanolic	19.48 ± 3.96
	Aqueous	94.54 ± 4.08
Suregada multiflorum (A. Juss) Baill.	Ethanolic	>100
	Aqueous	>100
Bauhinia strychnifolia Craib.	Ethanolic	28.11 ± 0.46
	Aqueous	50.06 ± 3.76
Caesalpinia sappan Linn.	Ethanolic	5.75 ± 0.86
	Aqueous	30.23 ± 3.16
Andrographis paniculata (Burm.f.)	Ethanolic	>100
Wall. Ex Nees.Aqueous	>100	
Coscinium fenestratum (Gaertn.) Colebr.	Ethanolic	>100
	Aqueous	>100
Samannachan remedy	Ethanolic	31.45 ± 4.92
	Aqueous	47.18 ± 4.74
Propyl gallate		9.46 ± 2.52
(Positive control)		

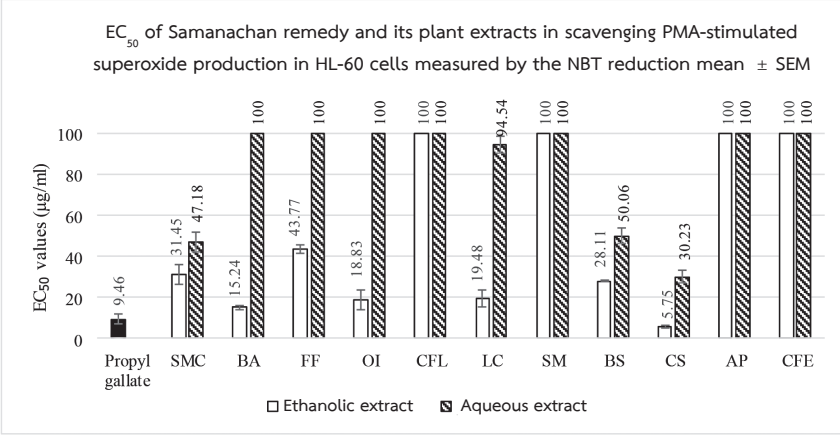

Figure 1 EC_{50} value of Samanachan remedy and its plant ingredients extracts in scavenging PMA-stimulated superoxide production in HL-60 cells measured by the NBT reduction (mean ± SEM), (n = 3).

Analysis of the ethanolic extract of Samanachan remedy for its chemical composition by HPLC technique, showed that brazilin was the main compound of Samanachan extract and it showed highest peak area of HPLC chromatogram. The chemical structure of brazilin as shown in Figure 2. Chromatogram of ethanolic extract of Samanachan remedy, as showed in Figure 3.

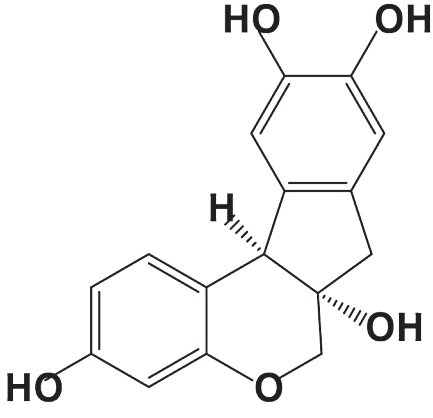

Figure 2 Chemical structure of brazilin.

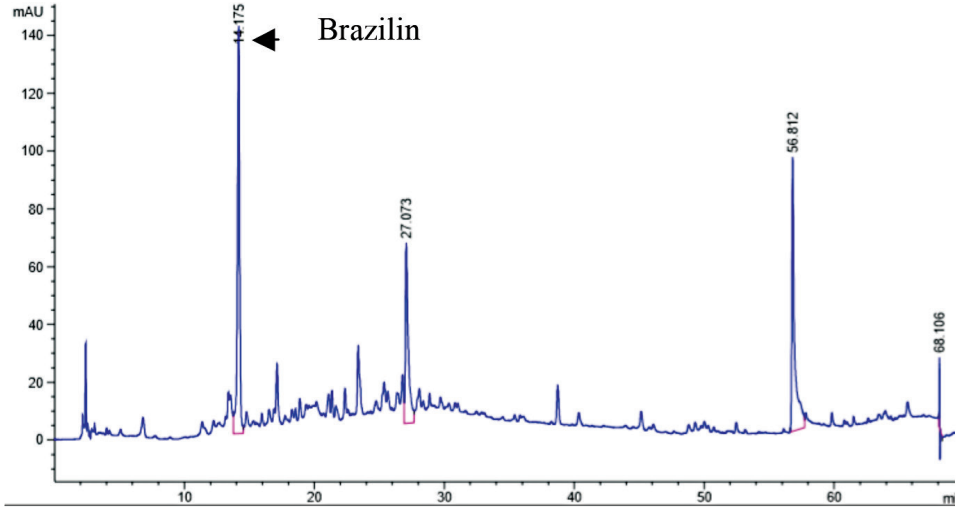

Figure 3 Chromatogram of ethanolic extract of Samanachan remedy, the wavelength at 254.8 nm.

Discussion

Samanachan (SMC) is one of the remedies used for cancer patients in Khampramong temple. It is used as an adaptogenic drug. Water is the better solvent to obtain high extraction yield of Samanachan remedy that may contain polar substances. However, water extract and ethanolic extract of this remedy both showed moderate antioxidant activity by inhibition of superoxide formation as compared to the positive control, but the ethanolic extract showed better antioxidant activity than the aqueous extract. One possibility is substances that have low polarity or moderate polarity may show antioxidant activity higher that than high polarity substances. *Betula alnoides* is the highest proportion in remedy (28.57%) and showed EC_{50} value of ethanolic extract 15.24 ± 1.16 µg/ml. *B. alnoides* has been reported that the EC_{50} value of its 80% methanolic and aqueous extracts have been reported by Ghimire *at al.* (2017) as 80.68 ± 1.71% and 76.28 ± 2.33%, respectively on free radical scavenging activity.[11] This difference in this result for *Betula alnoides* may be due to a different assay being used to determine antioxidant activity. The ethanolic extract of *Caesalpinia sappan* showed the highest antioxidant activity by NBT assay with EC_{50} value (5.75 ± 0.86 µg/ml) which was higher activity than propyl gallate as positive control but the proportion of this herb in SMC is low (8.57%). Investigation of *Caesalpinia sappan* wood resulted in the isolation of various structural types of phenolic components including one xanthone, one coumarin, three chalcones, two flavones three homoisoflavonoids and brazilin. Brazilin, is a major and active compound found in *Caesalpinia sappan* heartwood and has shown various biological activities including antioxidant, anti-inflammation and hepatoprotective properties.[12] From the Samanachan extract chromatogram, brazilin appeared at 14.75 min and the content of brazilin was found to be 0.14 ± 0.01 mg/g of extract. Brazilin has been reported for its antioxidant activity by DPPH radical scavenging assay (IC_{50} = 57.2 µg/ml) and ferric reduction activity as compared to standard

vitamin E.[13] Brazilin has also shown dose-dependent inhibition of peroxide formation in linoleic acid emulsion during incubation at 50 °C for 250 h. Also, Brazilin has shown IC_{50} value of 28.8 µg/ml as determined by ABTS radical scavenging activity.[14]

These results support the Thai traditional use of Samanachan remedy in Arokhayasala, Khampramong temple in that this remedy showed moderate antioxidant activity by an inhibitory effect on superoxide reduction by NBT assay. The brazilin is a main active antioxidant compound of this remedy. Further studies on the antioxidant activity of this remedy should be studied in animal model because it should be helpful in confirming its antioxidant effect.

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บทคัดย่อ

ฤทธิ์ต้านอนุมูลอิสระในเซลล์ ของตำรับยาสมานฉันท์ และสมุนไพรเดี่ยวในตำรับ

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บทนำ: ตำรับยาสมานฉันท์เป็นตำรับยาที่ใช้เพื่อปรับสมดุลของธาตุต่างๆในร่างกายของผู้ป่วยมะเร็งที่วัดคำประมง จังหวัดสกลนคร โดยมีสมุนไพรที่เป็นส่วนประกอบตำรับทั้งหมด 10 ชนิด ได้แก่ กำลังเสือโคร่ง ม้ากระทืบโรง ช้างน้าว กระจ้อนเน่า ตะไคร้ต้น ขันทองพยาบาท ย่านางแดง ฝาง ฟ้าทะลายโจร และ แฮ่ม

วิธีการศึกษา: สมุนไพรทั้งหมดในตำรับถูกสกัดด้วย 2 วิธี คือ การหมักด้วย 95% เอทานอล และการต้มกับน้ำ จากนั้นสารสกัดทั้งหมดจะถูกนำไปทดสอบฤทธิ์ต้านอนุมูลอิสระในเซลล์มะเร็ง HL-60 โดยศึกษาการยับยั้งซูเปอร์ออกไซด์ด้วยวิธี NBT assay

ผลการศึกษา: สารสกัดตำรับสมานฉันท์ชั้นเอทานอล และชั้นน้ำสามารถยับยั้งการเกิดซูเปอร์ออกไซด์ ได้ที่ IC_{50} เท่ากับ 31.45 ± 4.92 µg/ml และ 47.18 ± 4.74 µg/ml ตามลำดับ โดยสารสกัดเอทานอลของฝางมีฤทธิ์ต้านอนุมูลอิสระดีที่สุด (IC_{50} 5.75 ± 0.86 µg/ml) เมื่อเปรียบเทียบกับสารสกัดอื่นๆที่นำมาทดสอบ

สรุปผลการศึกษา: สารสกัดตำรับสมานฉันท์ชั้นเอทานอล และชั้นน้ำมีฤทธิ์ในการต้านอนุมูลอิสระระดับปานกลางเมื่อเปรียบเทียบกับสารมาตรฐาน

คำสำคัญ: ตำรับยาสมานฉันท์, การต้านอนุมูลอิสระ, วัดคำประมง, ตำรับยาสมุนไพรไทย, มะเร็ง, ยับยั้งซูเปอร์ออกไซด์