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RESEARCH ARTICLE

In vitro Antibacterial, Cytotoxic and Antioxidant Activities of Thai Traditional Medicine Called Trikatuk Remedy for Oral Cancer

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Abstract

Oral cancer is commonly observed in patients with head and neck cancers. Efforts to identify novel antibacterial, cytotoxic and antioxidant agents that can prevent these diseases are ongoing. The 'Trikatuk' remedy, composed of 3 herbs-pepper (*Piper nigrum* Linn), long pepper (*Piper retrofractum*), and ginger (*Zingiber officinale* Roscoe) was recorded in Thai scripture as a remedy for diseases of the mouth and throat. Therefore, the present study investigated its antibacterial activity via the disc diffusion method, cytotoxic activity via the sulforhodamine B assay, and antioxidant activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation decolourisation, and ferric ion reducing antioxidant power (FRAP) assays. The phytochemical composition of the extracts was analyzed using high-performance liquid chromatography (HPLC). The results showed that the 95% ethanolic extract from Trikatuk remedy did not inhibit the growth of *Aggregatibacter actinomycetemcomitans*, *Staphylococcus aureus*, and *Streptococcus mutans*. In contrast, the Trikatuk remedy showed high cytotoxic activity and very strong antioxidant activity with IC_{50} values of 30.68 and 25.21 µg/mL, respectively. Piperine, 6-gingerol and 6-shogaol were the major compounds in the ethanolic extract of Trikatuk remedy, found 223.43, 11.85 and 7.67 mg/g of extract, respectively. In conclusion, the data presented in this study revealed its cytotoxic and antioxidant properties, which correspond to the traditional Thai theory of using herbal recipes to treat various diseases.

Keywords: Thai traditional medicine, Trikatuk remedy, Antibacterial activity, Cytotoxic activity, Antioxidant activity, HPLC

Introduction

Head and neck cancer (HNC) is known to be the sixth most common malignancy globally [1]. Oral cancer constitutes 48% of head and neck cancer cases [2]. Asia bears the highest burden of oral cancer amongst all continents with cases and mortality rates reaching 64.2% and 73.3% respectively [1]. Southeast Asia, oral cancer is ranked as the fourth most common cancer within the population, with 149,102 new cases reported in 2018 [1]. According to cancer reports from the National Cancer Institute of Thailand, from 2004 to 2015, the incidence of oral cancer increased from 4.8 to 5.5 per 100,000 population in men and from 3.6 to 4.3 per 100,000 population in women [3]. In 2020, the prevalence of oral cancer in Thailand was 18.7% [4]. Moreover, ninety percent of oral cancer cases are histologically diagnosed as oral squamous cell carcinomas (OSCCs) [2].

Although the etiology of 70% - 80% of oral cancers has been primarily linked to tobacco chewing, smoking, and alcohol consumption, other factors like an individual's genetic susceptibility, and external agents such as dietary factors may exert synergistic role in tumorigenesis. Notably, approximately 15% of oral cancer patients have no known risk factors and the disease in this population may pursue a particularly aggressive course. This can be attributed to infections involving viruses such as the human papilloma viruses (HPV) and Epstein Barr Virus (EBV), fungi such as *Candida albicans*, and certain bacteria [5]. Pathogens that affect cancer development in the gastrointestinal tract are also found in the oral cavity. Some specific species strongly correlate with oral cancer, such as *Streptococcus sp*., *Peptostreptococcus sp*., *Prevotella sp*., *Fusobacterium sp*., *Porphyromonas gingivalis*, Capnocytophaga *gingivalis* [6] and Aggregatibacter *actinomycetemcomitans* [7].

However, the incidence of oral cancer is not very high compared to that of other cancers such as breast, colorectal, cervical, and lung cancer. Nevertheless, patients often visit a doctor for the first time when the disease has already progressed significantly, spreading to other organs, and requiring urgent and continuous treatment. Advanced oral cancer necessitates complex and invasive treatments that directly affect food consumption and speech, thereby greatly reducing patients' daily quality of life [3]. Despite new management strategies, the 5-year survival rate of oral cancer is still below 50% in most countries [2]. Owing to the poor outcomes of oral cancer, prevention is necessary.

According to traditional Thai medicine, the elements that make up human life and maintain a healthy body are earth, water, wind, and fire. If any one of these elements within the body loses its function or functions abnormally, it can lead to health problems [8]. These imbalances are linked to internal and external factors. There is a Thai scripture called 'Mook ka-Roke' that describes diseases occurring in the mouth and throat, along with the use of herbal medicines for treatment [9]. The herbal formulas recorded in this text are divided into 2 groups: Those that can be used to treat all diseases and those that are specific to each type of disease. Notably, the 'Trikatuk' remedy, composed of 3 herbs -pepper (*Piper nigrum* Linn), long pepper (*Piper retrofractum*), and ginger (*Zingiber officinale* Roscoe) is a formula that can treat all diseases of the mouth and throat [9].

From the literature review, it was found that the Trikatuk remedy and its individual herbs possess diverse biological effects. For example, the 80% ethanol extract of Trikatuk remedy showed toxicity against HepG2 liver cancer cells with a half-maximal cytotoxic concentration (CC_{50}) value of 26.5 ± 3.9 µg/mL [10]. Furthermore, the Trikatuk remedy can inhibit the cell cycle of cholangiocarcinoma cells through the action of CDK2, p21, and p27 [11]. Additionally, the Trikatuk remedy has been found to have antioxidant [12,13], cholinesterase inhibitory [13], anti-inflammatory [14], and anti-allergic properties [15]. Pepper as the "King of spices" is a widely used spice which adds flavor of its own to dishes and enhances the taste of other ingredients. *P. nigrum* and its bioactive compounds were also found to possess important pharmacological properties including antimicrobial, antioxidant, anticancer, antidiabetic, anti-inflammatory, and the major bioactive compound is piperine [16]. Long pepper is a plant that is commonly found in Southeast Asia and is empirically used to treat various diseases. The pharmacological activity of *P.retrofractum* has been reported to have antimicrobial, antioxidant, cytotoxic, analgesic, androgenic,aphrodisiac, antihyperlipidemic, antihyperuricemic, lowering leukocyte count, antileishmanial and immunostimulant effects [17]. Ginger is a medicinal plant that has been widely used all over the world and is indigenous to tropical Asia. It provides health advantages for humans because of its biological activities such as antioxidants, anti-inflammation, antibacterial, antiviral, antifungal, antihyperlipidemic, antiobesity, anti-allergy and hepatoprotective activities. Gingerol compounds in Zingiber officinale consist of gingerol (23% - 25%), shagaol (18% - 20%), paradol (1% - 3%) and zerumbone (1% - 3%) [18].

However, there are no reports on its antibacterial activity against *Aggregatibacter actinomycetemcomitans*, *Staphylococcus aureus*, and *Streptococcus mutans*, which are associated with chronic periodontitis, and its cytotoxicity against oral cancer cells (KB cells). Additionally, the antioxidant activity and phytochemical composition of the ethanol extract of the remedy were evaluated. Therefore, this study aimed to provide empirical evidence that may lead to the development of a new alternative treatment to be used alongside anticancer drugs for the future treatment of oral cancer.

Materials and methods

Plant materials and extract preparation

In May 2023, ginger rhizomes, pepper seeds and long pepper fruit accompanied by a certificate of analysis, were purchased from a traditional herbal drug store in Nakhon Pathom Province. The voucher specimen of ginger rhizomes, pepper seeds and long pepper fruit were PBM 006621, PBM 006622, and PBM 006623, respectively were deposited by the Sireeruckhachati Nature Learning Park Project, Thailand. The voucher specimens were identified by Dr.Sunisa Sangvirotjanapat, researcher of Project of Institute Establishment for Sireeruckhachati Nature Learning Park, Mahidol University. Each individual sample was then cleaned and dried at 50 °C. The dried samples were mechanically ground into coarse powders. Next, each coarse powder was combined in equal proportions (1:1:1) by weight, macerated with 95% ethanol for 3 days, and filtered. This process was repeated twice with the residue. The resulting extracts were concentrated under reduced pressure using a rotary evaporator, and the percentage yield was calculated. The 95% ethanol crude extracts were stored at – 20 °C until testing.

Phytochemical compositions

Content of piperine, 6-gingerol and 6-shogaol

High-performance liquid chromatography (HPLC) was performed to determine the composition of piperine, 6-gingerol and 6-shogaol, following the method described by Pattanacharoenchai [19]. The chromatographic system utilised a C18 reverse-phase column (250×4.60 mm^2, 5 microns; Phenomenex, Inc., USA) protected by a Security Guard Cartridge (C18, 4×3.0 mm^2; Phenomenex, Inc., USA). The gradient mobile phase consisted of water (A) and acetonitrile (B) at the following ratios: 0 - 25 min, 60:40; 25 - 40 min, 50:50; 40 - 45 min, 5:95; 45 - 45.10 min, 0:100; 45.10 - 50 min, 60:40. The flow rate was 1 mL/min, and the peak response was detected using a diode array detector at an absorbance of 227 nm. The operating temperature was maintained at room temperature.

Standard solutions of piperine, 6-gingerol and 6-shogaol were prepared in methanol and serially diluted to construct calibration curves at concentrations of 1, 5, 10, 25, 50, 80 and 100 µg/mL. Sample solutions were prepared by dissolving the extract in methanol and sonicating for 10 min to produce an extract solution at a concentration of 10 mg/mL. All solutions were filtered through a 0.45 µm membrane filter prior to injection into the HPLC for determining their chemical content.

Determination of total phenolics content

Total phenolic content (TPC) was determined using the Folin-Ciocalteu assay (20). Trikatuk remedy and individual plant ingredient extracts were dissolved in absolute ethanol. The extract solution (20 µL) was added into a 96-well plate, and 100 µL of Folin-Ciocalteu's reagent was added and mixed. After standing for 5 min, 80 µL of sodium carbonatesolution (7.5% w/v) was added. The samples were then mixed and incubated at room temperature for 30 min. The absorbance was measured at 765 nm using a spectrophotometer. Standard solutions of gallic acid (2.5, 5, 10, 20, 40, 80 and 100 µg/mL) were prepared, and a calibration curve was generated. TPC was calculated against the calibration curve. The results are expressed as milligrams of gallic acid equivalents per gram of sample (mg GAE/g). All measurements were performed in triplicates.

Cell culture

KB (HeLa derivative) cells (ATCC CCL17) were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Minimum Essential Medium (MEM) supplemented with 10% heated foetal bovine serum, 50 IU/mL penicillin and 50 µg/mL streptomycin. KB cells were seeded into 96-well plates at an initial concentration of 3×10^3 cells/well in 100 µL of medium. The cells were maintained at 37 °C in a 75% relative humidified atmosphere with 5% CO_2 for 24 h. After 24 h. the cells were treated with various extract concentrations (1, 10, 50 or 100 µg/mL) in complete medium for 72 h. The medium was then replaced with 200 µL of fresh medium, and the cells were cultured for an additional 72 h. Following this incubation, the viability of all cells was measured. The technique and dose selection were described following the procedure of Itharat *et al.* [21,22].

Cytotoxic activity

A sulfordoramine B (SRB, Sigma-Aldrich, St. Louis, MO, USA) assay was preformed to measure cell viability [23]. The viable cells were fixed with 100 µL of 40% (w/v) trichloroacetic acid (Q-Rec, New Zealand) at 4 °C for 1 h and then stained with 50 µL of 0.4% (w/v) SRB solution in 1% acetic acid (Sigma-Aldrich, St. Louis, MO, USA) for 30 min. After staining, the colour was dissolved with 10 mM Tris base [Tris(hydroxymethyl)aminomethane] (Sigma-Aldrich, St. Louis, MO, USA). Absorbance was measured at 492 nm. using a microplate reader (Thermo Scientific). The percentage inhibition was calculated using the formulae below:

$$\text{Percentage Inhibition} = [\frac{\text{Abs. control} - \text{Abs. sample}}{\text{Abs. control}}] \times 100$$

The half-maximal inhibitory concentration (IC_{50}) was calculated using GraphPad Prism 9 software (GraphPad Software Inc., San Diego, CA, USA).

Determination of antimicrobial activity

Microorganisms

This study was approved by the Naresuan University Institutional Biosafety Committee, Thailand under Biosafety Level 2 (BSL II, NUIBC 048/2566). The following microorganisms were used in the present study: (i) *Aggregatibacter actinomycetemcomitans* (ATCC 43718), (ii) Staphylococcus aureus (ATCC 25923), and (iii) Streptococcus mutans (ATCC 25175)

Determination of antimicrobial activity using the disc diffusion method

This method was used to screen the antimicrobial activity of the extracts [24]. Filter paper discs (6 mm in diameter) were impregnated with 10 µL of the extract (conc. 500 mg/mL). The air-dried discs were placed on inoculated Mueller–Hinton agar (MHA) surfaces. Chlorhexidine and Ciprofloxacin (conc. 1 mg/mL) were used as positive controls in this study. The plates were incubated at 37 °C for 24 h. The zone of inhibition was calculated by measuring its diameter. Three different fixed directions were measured in triplicate, and the average value was calculated.

Determination of minimum inhibitory concentration (MIC)

The MIC value was determined by a broth dilution method [25]. Briefly, all extracts were dissolved in 2% dimethylsulfoxide (DMSO) in which the maximum final concentration was 40 mg/mL. The serial 2-fold dilution was performed in a concentration range of 0.31 - 40.00 mg/mL in a 96-well plate (50 µL/well). Then, 50 µL of 0.5 McFarland of the tested microorganisms was added to each well and incubated at 37 °C for 24 h. After that, 10 µL of resazurin (1 mg/mL) was added into each well and incubated at 37 °C for 2 h. The final concentration that did not show a color change of resazurin from blue to pink was the MIC value.

Determination of minimum bactericidal concentration (MBC)

The MBC value is the lowest extract concentration of extract which can kill bacteria at least 99.9% within 24 h. In short, the contents in the MIC well and those above the MIC for each micro-organism was streaked on MHA and incubated at 37 °C for 24 h. After incubation, the bacteria colonies on MHA were observed. The final concentration that bacteria colony did not appear was the MBC value [25].

Determination of antioxidant activity

Determination of antioxidant activity using the DPPH radical scavenging assay.

Antioxidant activity was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH), according to the modified method of Yamasaki *et al*. [26]. In brief, 180 µL of 6×10^{-5} M DPPH solution in absolute ethanol was mixed with 20 µL of different concentrations of the extract and incubated for 30 min in dark at room temperature. The absorbance was measured at 520 nm using a microplate reader. Quercetin was used as a positive control. Initially, DPPH half maximal effective concentration (EC_{50}) was determined and defined as the antioxidant concentration needed to reduce DPPH activity by 50%, a parameter widely used to measure antioxidant activity. The antioxidant scavenging activity was calculated as a percentage inhibition using the formula below:

$$\text{Percentage inhibition} = [\frac{\text{Abs.control} - \text{Abs.sample}}{\text{Abs.control}}] \times 100$$

Determination of antioxidant activity using ABTS radical cation decolorization assay

2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assay was conducted according to the method of Re *et al*. [27]. The ABTS assay involved 2 steps: Preparation of the ABTS radical solution, followed by the discoloration assay. ABTS radical solution was prepared by mixing 7 mM ABTS solution with 2.45 mM potassium persulfate. The reaction mixture was allowed to stand in the dark at room temperature for 12 - 16 h to complete radical generation. Then, the ABTS radical solution was diluted with deionised water to reach an absorbance of 0.68 - 0.72 at 734 nm before being used. The discolouration assay was assessed by mixing 180 µL of ABTS radical solution with 20 µL of various dilutions of each extract in 96 well microplates. The reaction mixture was allowed to stand for 6 min, and the absorbance was measured at 734 nm using a microplate reader. Trolox was used as a positive control. The scavenging activity of extracts against ABTS radicals was calculated by the percentage inhibition of scavenging activity using the formula below and expressed as EC_{50} (μg/mL).

$$\text{Percentage inhibition} = [\frac{\text{Abs.control} - \text{Abs.sample}}{\text{Abs.control}}] \times 100$$

Determination of antioxidant activity using ferric ion reducing antioxidant power assay (FRAP)

FRAP was evaluated by measuring the Fe (III)/TPTZ - complex using a colourimetric method with a spectrophotometre [28]. The FRAP reagent was prepared using 10 ml of acetate buffer (300 mM), adjusted to pH 3.6 by the addition of acetic acid. This was then combined with 1 mL of 10 mM 2, 4, 6-tripyridyls-triazine (TPTZ) in 40 mM HCl and 1 mL of 20 mM ferric chloride hexahydrate dissolved in distilled water. These components were mixed in a 10:1:1 ratio respectively. The FRAP reagent was prepared fresh daily and warmed to 37 °C for 4 min in a water bath. Approximately 10 mg of crude extracts was weighed into centrifuge tubes and diluted to 1 mg/mL. Aliquots of sample solutions (20 µL) were added to 180 µL of the FRAP reagent, mixed well and allowed to stand at room temperature for 8 min. Absorbance was measured at 593 nm. The increased absorbance of the reaction mixture indicated increased reducing power of the sample. The results were expressed as mg Fe (II) per g of the extract sample.

Statistical analysis

All data are presented as mean ± standard error of the mean (SEM) in triplicate. One-way analysis of variance (ANOVA) with Dunnett's test, was used to compare sample groups and positive controls; a *p*-value < 0.05 was considered significant. Statistical analyses were performed using GraphPad Prism 9 software.

Results and discussion

Extract preparation

The 95% ethanolic extract of the Trikatuk remedy, yielded 7.22% (w/w). The percentage yield of all individual ethanolic extracts from the plant ingredients ranged from 3.98% - 9.83%. *P. retrofractum* had the highest percentage yield of ethanolic extract, while *Z. officinale* had the lowest. These results are summarized in **Table 1**.

Table 1 The percentage yield of Trikatuk remedy and its plant components.

Scientific name	Common name	Used Part	Code	% Yield
Piper nigrum Linn	Pepper	Fruit	PN	6.32
Piper retrofractum	Long pepper	Fruit	PR	9.83
Zingiber officinale Roscoe	Black tulsi	Rhizome	ZO	3.98
-	Tri-Ka-Tuk remedy	-	TK	7.22

Phytochemical compositions

Piperine, 6-gingerol, and 6-shogaol contents in the ethanolic extract of the Trikatuk remedy were evaluated using HPLC analysis. The mean contents of piperine, 6-gingerol and 6-shogaol in the Trikatuk remedy were 223.43, 11.85 and 7.67 mg/g of extract, respectively.

The TPC of all extracts were determined as milligram of gallic acid equivalent per gram of each extract (mg GAE/g). *Z. officinale* showed the highest TPC with a value of 197.64 mg GAE/g, followed by the Trikatuk remedy with 95.20 mg GAE/g). *P. retrofractum* showed the lowest, with 22.69 mg GAE/g.

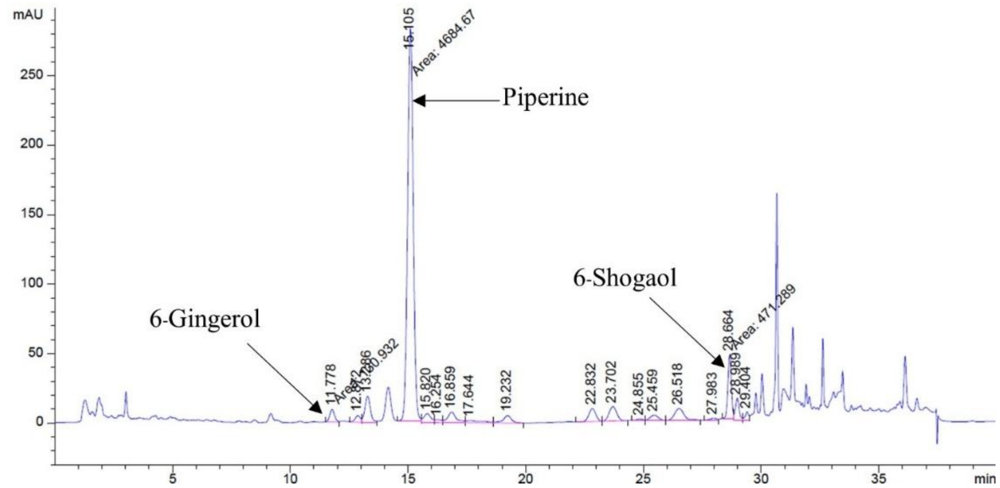

Figure 1 HPLC chromatogram of the 95% ethanolic extract of Trikatuk remedy.

Cytotoxic activity

The results of cytotoxic activity demonstrate that the Trikatuk remedy showed high cytotoxic activity on KB (HeLa derivative) cell lines, with an IC_{50} value of 30.68 µg/mL. Similarly, *Z. officinale* exhibited high cytotoxic activity with an IC_{50} value of 21.19 µg/mL, and the other extracts also demonstrated high cytotoxic activity. The results are summarised in **Table 2**.

According to the criteria described by the National Cancer Institute (NCI, USA), crude extracts are considered to possess high cytotoxic activity with IC_{50} values below 30 µg/ml and moderate activity with IC_{50} values below 50 µg/mL [29]. The result of this study demonstrates that the Trikatuk remedy and all plant ingredient extracts showed high cytotoxic activity. In particular, *Z. officinale* exhibited high cytotoxicity, with an IC_{50} value of 21.19 µg/mL. These results suggest that the extracts with an IC_{50} below 30 µg/mL exhibited significant cytotoxicity. This finding is consistent with previous studies [10]. The ethanolic extract of Trikatuk possesses anticarcinogenic activity, due to at least 2 cytotoxic components: Piperine, and 6-gingerol [30]. This implies that the ethanolic extracts from Trikatuk displayed selective toxicity towards cancer cell lines, likely due to the contribution of antitumor or anticancer compounds. This finding suggests that the cytotoxicity of these recipes may be caused by toxic compounds deposited in the plants.

Table 2 Cytotoxic activity of Trikatuk remedy on the KB (HeLa derivative) cells (ATCC CCL17) (n = 3).

Sample	Inhibition (%) of at various concentrations (µg/mL)				IC_{50} ± SEM (µg/mL)
	1	10	50	100	
PN	4.97± 0.68	20.27± 2.55	72.57± 2.22	99.78 ± 0.18	30.06 ± 3.19
PR	11.49 ± 2.16	25.32 ± 1.44	83.37 ± 0.45	99.76 ± 0.13	25.63 ± 0.46
ZO	6.48 ± 1.12	26.96 ± 0.63	93.01 ± 0.96	99.80 ± 0.21	21.19 ± 0.82
TK	5.44 ± 2.22	18.67 ± 1.24	75.97 ± 2.29	99.73 ± 0.23	30.68 ± 1.27

Antimicrobial activity

The ethanolic extract of the Trikatuk remedy and all plant ingredients did not inhibit the growth of *A.actinomycetemcomitans, S. aureus and S. mutans.* The results are shown in **Table 3**.

All extracts were tested for antibacterial activity by using a microtiter plate-based method to confirm their antibacterial activity. The *P. nigrum* extract showed the best antibacterial activity against *A.actinomycetemcomitans, S. aureus and S. mutans* with MIC values of 5, 10 and 5 mg/mL and against *S. aureus* with MBC values of 40 mg/mL. The Trikatuk remedy, *P.retrofractum and Z.officinale* displayed MIC values of 20 mg/mL of all microorganisms and the Trikatuk remedy against *S. aureus* with MBC values of 40 mg/mL, as shown in **Table 4**.

Table 3 Inhibition zone (mm.) on 3 microorganisms of Trikatuk remedy (n = 3).

Sample	***Aa*** **(ATCC 43718)**	***S. aureus*** **(ATCC 25923)**	***S. mutans*** **(ATCC 25175)**
PN	NI	NI	NI
PR	NI	NI	NI
ZO	NI	NI	NI
TK	NI	NI	NI
Chlorhexidine	1.6	-	1.7
Ciprofloxacin	-	2.8	-

NI = No Inhibition, − = Not test

Table 4 The value MIC and MBC concentration of Trikatuk remedy extract (n = 3).

Sample	***Aa*** **(ATCC 43718)**		***S. aureus*** **(ATCC 25923)**		**S. mutans (ATCC 25175)**	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
PN (mg/mL)	5	> 40	10	40	5	> 40
PR (mg/mL)	20	> 40	20	> 40	20	> 40
ZO (mg/mL)	20	> 40	20	40	20	> 40
TK (mg/mL)	20	> 40	20	40	20	> 40
Ampicillin (ug/mL)	-	-	0.195	>50	-	-
Chlorhexidine (ug/mL)	6.25	> 50	-	-	12.5	> 50

NI = No Inhibition, - = Not test

In the present study, we screened the extracts of the Trikatuk remedy and all plant ingredients for their antimicrobial action against Gram-positive and Gram-negative bacteria. *A. actinomycetemcomitans* is an opportunistic Gram-negative periodontopathogen strongly associated with periodontitis and infective endocarditis. Recent evidence suggests that periodontopathogens can influence the initiation and progression of oral squamous cell carcinoma (OSCC) [31]. *S. mutans and S. aureus* are Gram-positive bacteria commonly found in the mouth, and both have been linked to oral cancer development and progression. *S. mutans* are the primary cause of dental caries but can also promote tumor growth and metastasis in OSCC [32]. *S. aureus*, which is less directly linked to OSCC, can induce the COX-2 dependent proliferation and malignant transformation of oral keratinocytes (32). Our results showed no zone of inhibition for any type of bacterium**.** These results are consistent with a previous report which found the ethanolic extracts of *Z. officinale*, *P. nigrum*, *P. retrofractum* and pure compounds, such as piperine, gingerol, and shogaol, could not protect against Gram- negative bacteria, such as *Escherichia coli*, *Salmonella spp.*, and *Salmonella typhi* (24). However, the interpretation of antimicrobial results requires greater scrutiny. Drawing conclusions about the absence of antimicrobial activity based solely on disc diffusion method is inadequate, as it fails to consider critical factors such as extract diffusion capacity in agar medium. As a result, we were tested for antibacterial activity by using a microtiter plate-based method to confirm their antibacterial activity. The results show that the Trikatuk remedy and all plant ingredients can inhibit 3 bacteria and can kill *S. aureus* with high concentrations of extract.

Antioxidant activity

For the DPPH radical scavenging assay, the positive control (BHT, butylated hydroxytoluene) showed an EC_{50} value of 13.78 µg/mL. *Z. officinale* demonstrated significantly more vigorous activity than the positive control, with an EC_{50} value of 11.83 µg/mL.

Although the Trikatuk remedy displayed good antioxidant activity, its value was lower than that of BHT. The results are summarised in **Table 5**.

The ABTS radical scavenging assay results of the extracts are also presented in **Table 5**. The positive control (BHT) exerted potent activity with an EC_{50} value of 5.55 µg/mL. This assay demonstrated that *Z. officinale* had excellent antioxidant activity, with an EC_{50} value of 10.86 µg/mL, while Trikatuk remedy extract showed an EC_{50} value of 42.45 µg/ mL.

For the FRAP value, the antioxidant activity of the extracts was calculated from standard curve of $FeSO_4$ and is presented in units of milligram Fe^{2+} per gram of extract (mg Fe^{2+} eq./g). The result found that the Trikatuk remedy showed antioxidant activity, with a FRAP value of 122.25 mg Fe (II)/g. The highest FRAP value among the plant extracts was that of *Z. officinale*, and the lowest was that of *P. retrofractum.*

The antioxidant capacity was evaluated using ABTS and DPPH assays, which are simple, rapid, inexpensive, acceptable, and widely used [33,34]. DPPH is the free radical most commonly used in antioxidant screening assays, where the capacity of each extract could be classified as very strong (IC_{50} < 50 µg/mL), strong (IC_{50} 50 - 100 µg/mL), moderate (IC_{50} 101 - 150 µg/mL), and weak (IC_{50} > 150 µg/mL) [35]. Overall, the Trikatuk formulations exhibited a very strong antioxidant capacity. Of the component herbs, only *Z. officinale* was classified as very strong, whereas *P. nigrum* and *P. retrofractum* exhibited weak antioxidant capacities. The results from the DPPH assay were similar to those from the ABTS assay. *Z. officinale*, which had the highest TPC also, exhibited the strongest ABTS and DPPH radical scavenging capacities. Similar to our results, many studies have consistently reported a correlation between the antioxidant activity estimated by ABTS and DPPH assays and TPC [36-38]. This suggests that *Z. officinale*, which has the highest TPC, could be the main contributor to the antioxidant function of the Trikatuk remedy.

Table 5 Antioxidant activity, FRAP value, and total phenolic content of Trikatuk remedy and its plant components (n = 3).

Sample	Antioxidant activity (EC_{50} µg/mL)		FRAP value (mg Fe (II)/g)	Total phenolic content (mgGAE/g)
	DPPH	ABTS		
PN	56.57 ± 1.09	62.45 ± 1.78	92.25 ± 1.36	40.65 ± 0.04
PR	> 100	> 100	51.77 ± 4.80	22.69 ± 2.56
ZO	11.83 ± 0.71	10.86 ± 1.43	764.00 ± 12.40	197.64 ± 4.90
TK	25.21 ± 4.67	42.45 ± 1.78	122.25 ± 1.65	95.20 ± 0.75
BHT	13.78 ± 0.23	5.55 ± 0.70	661.48 ± 20.32	NT

Correlation analysis

The correlation analysis was evaluated from the correlation coefficient (R) of the best fit regression model between 2 variables. The correlation between cytotoxic activity, DPPH, ABTS, FRAP value and total phenolic content shown highly related. The results are shown in **Figures (2) - (5)**. The correlation coefficient of DPPH and total phenolic content presents the highest relationship with the value of 0.9995. The correlation between the FRAP assay, ABTS, cytotoxic activity and the total phenolic content with the R value of 0.9975, 0.9760 and 0.9720, respectively.

The correlation between the cytotoxic activity, antioxidant activity of plant ingredients and the total phenolic content was also evaluated. DPPH and ABTS scavenging activities were correlated to the total phenolic content with exponential regression model in negative direction. The cytotoxic activity and FRAP assay correlated to total phenolic content with polynomial regression model. All correlation coefficients were more than 0.7 (in all positive and negative directions) which showed high correlation between 2 variables [39]. The correlation results demonstrated that the extracts with higher phenolic contents showed stronger cytotoxic activity and antioxidant activities.

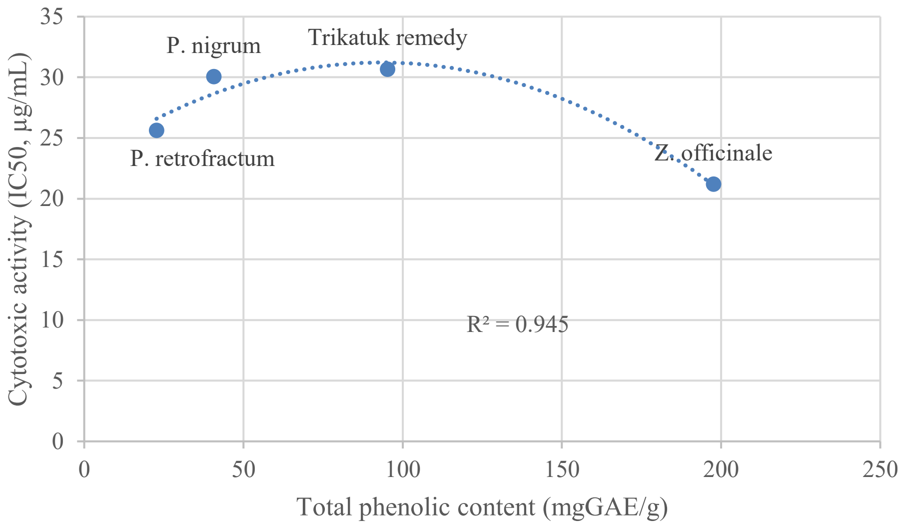

Figure 2 Correlation between cytotoxic activity and total phenolic content.

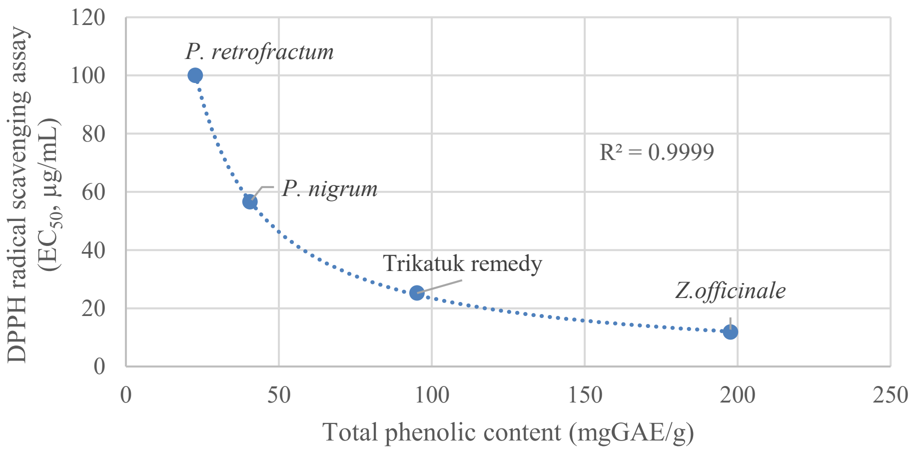

Figure 3 Correlation between DPPH scavenging activity and total phenolic content.

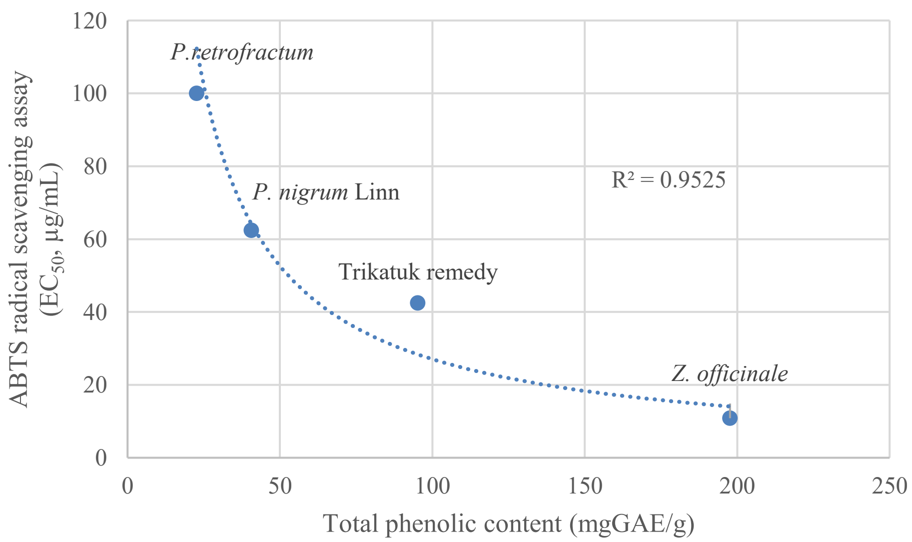

Figure 4 Correlation between ABTS scavenging activity and total phenolic content.

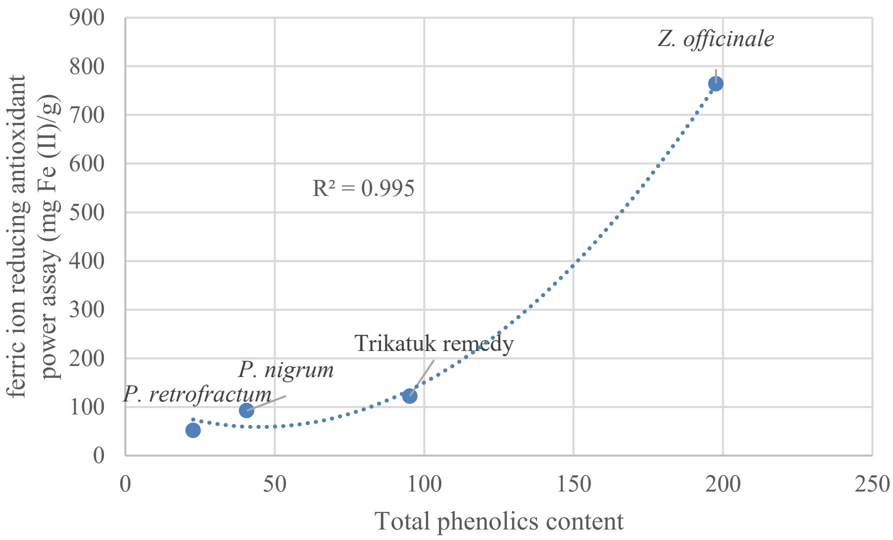

Figure 5 Correlation between FRAP assay and total phenolics content.

Conclusions

This study demonstrates that the Trikatuk remedy has significant cytotoxic and antioxidant activities. Furthermore, this is the first report on its antibacterial activity against oral bacteria. In addition, the data presented in this study revealed the cytotoxic and antioxidant properties, which correspond to the traditional Thai theory of using herbal recipes to treat various diseases. The limitations of this study include incomplete study in cancer cells and bacterial cells related to cancer pathogenesis. Therefore, the next study should expand the study in oral cancer cells and normal cells or add *Porphyromonas gingivalis* or *Tannerella forsythia*, which are bacteria related to oral cancer pathogenesis. Moreover, further investigation into its mechanism of action and pharmacological activities is needed for future healthcare benefits. Therefore, further studies on other biological activities, focusing on their mechanisms of action and pharmacological properties, should be undertaken to support animal model experiments and clinical studies.

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Declaration of generative AI in scientific writing

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CRediT author statement

Napaporn Pattanacharoenchai: Methodology; Investigation; Resources Data curation; Validation. **Rodsarin Yamprasert:** Conceptualization; Investigation; Writing - Original draft preparation; Writing – Review and Editing; Visualisation.

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