Investigation of Antitubercular and Cytotoxic Activities of Fruit Extract and Isolated Compounds from *Piper retrofractum* Vahl

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Abstract

A bioassay guided fractionation procedure used on the crude methanol extract of *Piper retrofractum* fruit identified 4 compounds: piperine, methyl piperate, sylvatine, and piperlonguminine. The bioactivities of 4 compounds and the crude methanol extract were examined. The crude methanol extract showed antibacterial activity against *Mycobacterium tuberculosis*, with a minimum inhibitory concentration (MIC) of 25.00 μg/mL. Piperine and piperlonguminine inhibited *M. tuberculosis* with MIC values of 50.00 μg/mL, whereas methyl piperate and sylvatine were inactive (% inhibition less than 90 % at a 50.00 μg/mL concentration). The crude extract and all isolated compounds were tested for cytotoxic activity against lung cancer cells (SCLC-H22 and NCI-H187) and human gingival fibroblasts (HGF) in cell cultures. All 4 isolated compounds showed weak cytotoxic activity against lung cancer cells (SCLC-H22 and NCI-H187) and human gingival fibroblasts (HGF) (% inhibition less than 20 % at a 5 μg/mL concentration, or a % inhibition less than 50 % at a 50 μg/mL concentration). However the crude methanol extract showed moderate cytotoxic behavior against lung cancer cells (NCI-H187) and human gingival fibroblasts (HGF), with an IC_{50} of 20.98 μg/mL and % inhibition = 56.83 at 25 μg/mL concentration, respectively. The present findings suggest that the clinical advantages of the crude extract and isolated compounds of *P. retrofractum* Vahl lie in combination therapy in order to enhance the efficacy of conventional antitubercular or anticancer drugs.

Keywords: *Piper retrofractum* Vahl, antitubercular, tuberculosis, cytotoxic activity

Introduction

Tuberculosis (TB) is contagious disease that normally attacks the respiratory system. It is caused by *Mycobacterium tuberculosis*, which mostly infects the lung alveoli and acts as a single infective agent. TB is responsible for the highest morbidity rate due to infection after HIV/AIDS. In 2014, an estimated 9.6 million people fell ill with TB, and 1.5 million died as a result of infection. The highest number of new TB cases has been reported in South-East Asia and the Western Pacific regions (58 %), with Africa accounting for 28 % of the total. India, Indonesia and China account for 23, 10 and 10 % of the global total of new cases, respectively [1]. Thailand is classified by WHO as one of the 22 countries in the world with the highest TB burden. With a population of nearly 67 million, Thailand has about 93,000 new cases each year, and an overall estimated TB prevalence of nearly 130,000 cases. 16 percent of them are also HIV positive. These figures are further complicated by the growing threat of drug resistant TB [2]. Long term *M. tuberculosis* infection results in injury to the alveolar epithelial cells, and loss of epithelial cells due to ongoing chronic inflammation [3] may eventually induce lung cancer [4].

The plant *Piper retrofractum* Vahl, or *Piper chaba* Hunt., is a member of the Piperaceae family, and is commonly used as a spice in cooking. The plant is found in many parts of Bangladesh, Indonesia, Malaysia, Vietnam, and Thailand, where it is used as a source of medicinal compounds to treat various conditions. The stem can be used to relieve pain in postnatal women and arthritis pain, and can also be used to treat diarrhea [5]. The leaves are used to treat carotid arterial disease and tendon discomfort. The root is applied to treat paresis, yaws, and diarrhea, and is used as an antipyretic and carminativ*e*. *P. retrofractum* is known in Thai as "Dee-Plee", and has been categorized nationally as an essential drug for the treatment of the gastrointestinal system. The fruit is useful for treating bronchitis, bronchial asthma, and muscle pain [6]. Previous phytochemical studies of *P. retrofractum* have identified several types of compounds. Alkaloids and lignans are found in the stem bark [7,8], while chabamide, a piperine dimer isolated from the plant stems, has shown antibacterial activity against *M. tuberculosis*, with MIC values of 12.5 µg/mL [9]. However, the most useful part is their fruit. Several amides and related compounds have been isolated from the fruits that are widely used in herbal recipes [10,11]. Preliminary screening by our group revealed that the fruit extract *of P. retrofractum* was active against *M. tuberculosis*, with MIC of 25 µg/mL. The objective of this work was to confirm the potential of fruit extract and isolated compounds as antibacterial agents against *M. tuberculosis* and cytotoxic drugs for the treatment of lung cancer.

Materials and methods

Instruments

^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectra were recorded on a Varian Unity Inova 500 MHz NMR spectrometer, using residual solvent as an internal standard. Mass spectra were measured on a Thermofinnigan MAT 95 XL mass spectrometer. IR spectra were recorded on a Perkin Elmer FT-IR spectrum one spectrometer (using KBr disks). UV spectra were obtained on a spectronic Genesys 6 UV-visible spectrometer. Melting points were determined by Buchi MIA-21 apparatus. Silica gel 60 (Merck, 40 - 63 µm) and Sephadex LH-20 (Pharmacia) were used for column chromatography. Thin-layer chromatography (TLC) was performed on Merck silica gel 60 F254 aluminum sheets (250 µm thickness).

Plant materials and extraction

The fruits of *Piper retrofractum* Vahl were obtained from Saiburi Samunpai, Songkhla province, Thailand, and specimens (voucher number SKP 146160301) were stored in the herbarium, Faculty of Pharmaceutical Sciences, Prince of Songkla University. Crude extract was obtained by first grinding the fruits to a course powder. The powder (1.5 kg) was then macerated 3 times with methanol (5 L) at room temperature for 3 days to obtain a crude extract of 165.02 g.

Isolation of compounds from *P. retrofractum* Vahl

Compounds were isolated from crude extract using bioassay-guided fractionation based on activity against *M. tuberculosis*. In the first step, the crude methanol extract was separated by vacuum liquid chromatography (VLC). Crude extract (20.0 g) was mixed with silica gel 60 (ratio 1:1) and dried under vacuum. The extract was subsequently loaded onto a classical column and eluted stepwise using hexane (100 %) to ethyl acetate and methanol (ratio 1:1). The separated fractions were analyzed by TLC using hexane and ethyl acetate (ratio 7:3) as a mobile phase and fractions giving similar TLC patterns were combined. Four fractions (F1-F4) were separated by these means and assayed for antibacterial activity against *M. tuberculosis*.

Four fractions were obtained using VLC; F1 (0.2 g), F2 (1.3 g), F3 (5.2 g), and F4 (5.5 g), with F2 and F3 displaying antibacterial activity against *M. tuberculosis* (MIC, 25-50 µg/mL). Compound 1 (piperine 1.32 g) was separated from F3 by recrystallization using hexane and methanol in the ratio 1:2. Compound 2 (methyl piperate 63.0 mg) was separated from F2 by recrystallization using chloroform and methanol in the ratio 2:1. Compound 3 (sylvatine 15.7 mg) was separated from F2 by silica gel column chromatography using hexane and ethyl acetate in the ratio 7:3, Sephadex LH-20 column chromatography using chloroform and methanol in the ratio 4:6, and silica gel column chromatography using hexane and

ethyl acetate in the ratio 7:3. Compound 4 (piperlonguminine 24.6 mg) was separated from F3 by Sephadex LH-20 column chromatography using hexane and ethyl acetate in the ratio 1:1. The crystals were purified using ethyl acetate. The structures of piperine, methyl piperate, sylvatine, and piperlonguminine (**Figure 1**) were elucidated by comparison with melting points, UV, IR, MS, ^{1}H, and ^{13}C NMR spectral data, published previously [12-15].

Piperine (compound 1)

White crystal. *mp*.130 °C; UV (chloroform) λ_{max}: 343, 311 (sh), 263 (sh), 255 (sh) nm; IR (KBr, cm^{-1}): 3009, 2940, 1634, 1584, 1449, 1366, 1032; ^{1}H-NMR (500 MHz, CDCl$_3$): δ (ppm) 1.55 (4H, *m*, 2-CH$_2$-), 1.62 (2H, *m*, -CH$_2$-), 3.49 (2H, *brs*, -CH$_2$-), 3.60 (2H, *brs*, -CH$_2$-), 5.94 (2H, *s*, -O-CH$_2$-O), 6.40 (1H, *d*, *J* = 14.64 , =CH-), 6.70 (2H, *m*, 2=CH-), 6.75 (1H, *d*, *J* = 8.03, aromatic H), 6.86 (1H, *dd*, *J* = 8.03, 1.68, aromatic H), 6.95 (1H, *d*, *J* = 1.68, aromatic H), 7.40 (1H, *m*,=CH-); ^{13}C-NMR (125, CDCl$_3$): δ (ppm), 24.6 (-CH$_2$-), 25.5 (-CH$_2$-), 26.6 (-CH$_2$-), 43.1 (-CH$_2$-N-), 46.8 (-CH$_2$-N-), 101.2 (-O-CH$_2$-O-), 105.5, 108.4, 122.4, 130.9, 148.0, 148.1 (aromatic C), 119.9 (=CH-), 125.2 (=CH-), 138.1 (=CH-), 142.4(=CH-), 165.7 (N-C=O); EIMS *m/z*: 285.1 (70 %), 201.1 (100 %), 173.1 (35 %), 143.1 (26 %), 115.1 (72 %), 84.1 (16 %).

Methyl piperate (compound 2)

White crystal. *mp*. 146 °C; UV (chloroform) λ_{max}: 347, 312 (sh), 256 (sh) nm; IR (KBr, cm^{-1}):, 1707, 1617, 1607, 1493, 1448, 1265, 1243, 1172, 1140, 1039; ^{1}H-NMR (500 MHz, CDCl$_3$): δ (ppm) 3.70 (3H, *s*, OCH$_3$), 5.96 (2H, *s*, -O-CH$_2$-O), 5,94 (1H, *d*, *J* = 15.14, =CH-), 6.60 (1H, *dd*, *J* = 15.40, 10.98, =CH-), 6.70 (1H, *d*, *J* = 8.06, aromatic H), 6.80 (1H, *d*, *J* = 15.40, =CH-C), 6.90 (1H, *dd*, *J* = 8.06, 1.47, aromatic H), 6.97(1H, *d*, *J* = 1.47, aromatic H), 7.40 (1H, *dd*, *J* = 15.14, 10.98, =CH-); ^{13}C-NMR (125, CDCl$_3$): δ (ppm) 51.5 (OCH$_3$), 101.3 (-O-CH$_2$-O-), 105.8, 108.5, 122.9, 140.9, 148.2, 148.5 (aromatic C), 119.8 (=CH-), 124.4 (=CH-), 130.4 (=CH-), 144.9 (=CH-), 167.5 (O-C=O); EIMS *m/z*: 232.1 (83 %), 201.1 (10 %), 173.1 (100 %), 143.1 (19 %), 115.1(32 %).

Sylvatine (compound 3)

Sticky cream, *mp*. 112 °C; UV (methanol) λ_{max}: 228 (sh), 261, 305 (sh) nm; IR (KBr, cm^{-1}): 3304, 2923, 1656, 1628, 1615, 1255, 1043, 999, 963, 924, ^{1}H-NMR (500 MHz, CD$_3$OD): δ (ppm) 0.90 (6H, *d*, *J* = 6.80, 2CH$_3$), 1.36 (4H, *m*, 2-CH$_2$-), 1.45 (4H, *m*, 2-CH$_2$-), 1.80 (1H, *m*, -CH-), 2.16 (4H, *m*, 2-CH$_2$-), 3.05 (2H, *m*, N-CH$_2$-). 5.89 (2H, *s*, -O-CH$_2$-O), 5.90 (1H, *d*, *J* = 15.14, =CH-), 6.10 (2H, *m*, 2=CH-), 6.20 (1H, *m*, =CH-), 6.30 (1H, *m*, =CH-), 6.70 (1H, *d*, J = 8.60, aromatic H), 6.76 (1H, *dd*, *J* = 8.60, 1.70, aromatic H), 6.89 (1H, *d*, *J* = 1.70 ,aromatic H), 7.10 (1H, *m*, =CH-); ^{13}C-NMR (125, CD$_3$OD): δ (ppm) 20.4 (2CH$_3$), 29.7 (-CH-), 29.7 (-CH$_2$-), 29.9 (-CH$_2$-), 30.0 (-CH$_2$-), 30.1 (-CH$_2$-), 33.8 (-CH$_2$-), 33.9 (-CH$_2$-), 48.0 (N-CH$_2$-), 102.2 (-O-CH$_2$-O-), 106.2, 109.0, 121.2, 133.9, 148.0, 149.4 (aromatic C), 123.0 (=CH-), 129.9 (2=CH-), 130.8 (=CH-), 142.1 (=CH-), 143.9 (=CH-), 169.2 (N-C=O); EIMS *m/z*: 383.2 (68 %), 248.2 (88 %), 180.2 (36 %), 161.1 (48 %), 152.1 (66 %), 131.1 (100 %), 103.1(71 %).

Piperlonguminine (compound 4)

White powder. *mp*. 168 °C; UV (chloroform), λ_{max}: 340 nm; IR (KBr, cm^{-1}): 3289, 2958, 1644, 1615, 1552, 1445, 1371, 1254, 990; ^{1}H-NMR (500 MHz, CDCl$_3$): δ (ppm), 0.90 (6H, *d*, *J* = 6.50, 2-CH$_3$), 1.80 (1H, *m*, -CH-), 3.20 (2H, *dd*, *J* = 6.50, 6.00, -CH$_2$-), 5.58 (1H, *brs*, NH), 5.90 (1H, *d*, *J* = 15.00 , =CH-), 5.98(2H, *s*, -O-CH$_2$-O), 6.68 (1H, *dd*, *J* = 15.00, 11.00, =CH-), 6.75 (1H, *d*, *J* =15.00, =CH-) 6.76 (1H, *d*, 8.00 , aromatic H), 6.87 (1H, *dd*, *J* = 8.00, 1.50, aromatic H), 6.96 (1H, *d*, *J* = 1.50, aromatic H), 7.34 (1H, *dd*, *J* = 15.00, 11.00 , =CH-); ^{13}C-NMR (125, CDCl$_3$): δ (ppm) 20.1 (2CH$_3$), 28.6 (-CH-), 46.9 (-CH$_2$-N-) ,101.2 (-O-CH$_2$-O-), 105.6, 108.4, 123.1, 138.8, 148.1, 148.1 (aromatic C), 122.5 (=CH-), 124.6 (=CH-), 130.8 (=CH-), 141.0 (=CH-), 166.1 (N-C=O); EIMS *m/z*: 273.1 (72 %), 216.1 (21 %), 201.1 (100 %), 173.1 (68 %), 143.1 (31 %), 135.1 (51 %), 115.1 (68 %).

Assay of antibacterial activity of compounds isolated from *P. retrofractum* Vahl against *M. tuberculosis*

The antibacterial activity of piperine, methyl piperate, sylvatine, and piperlonguminine, respectively, was assessed by measuring the green fluorescent protein (GFP) produced by the GFP expressing *M. tuberculosis* strain H37Ra [16]. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of isolated compound that inhibited bacterial growth by 90 %.

H37Ra *M. tuberculosis* bacteria were cultivated on 7H10 agar containing 30 µg/ml kanamycin at 37 °C for 4 weeks or until growth was observed. The first step was to prepare by fully looping 2 - 3 single colonies into 7H9 broth supplement with 0.2 % v/v glycerol, 0.1 % w/v of casitone, 0.5 % v/v middlebrook OADC enrichment solution (BD Biosciences), and 30 µg/ml of kanamycin. The mixture was then incubated at 37 °C in a shaker incubator at 200 rpm until an optical density (OD) at 550 nm between 0.5 and 1 was obtained.

For batch cultivation, the starter culture was transferred at the rate of 1/10 volume to the 7H9 broth and incubated at 37 °C in the shaker incubator at 200 rpm until the OD at 550 nm was approximately 0.5 to 1. The bacteria was pelleted, washed, and then sonicated 8 times for 15 s each. The sonicated samples were aliquoted and frozen at −80 °C for a maximum period of 2 to 3 months prior to use.

Titer stocks were determined by colony forming unit (cfu) assay, and the seeding density for anti-TB assay was optimized by serial dilutions. The dilution that grew at a logarithmic phase on day 10 was used as an optimal bacterial seeding density. For assay in a 384-well format, the seeding was approximately 1×10^5 cfu/ml.

The assay was performed in quadruplicate wells, each well containing 5 µl of test samples serially diluted in 5 % dimethyl sulfoxide, followed by 45 µl of cell suspension. The plates were incubated at 37 °C for 10 day. Rifampicin, ofloxacin, streptomycin, isoniazid, and ethambutol were used as positive controls, and 0.5 % DMSO was used as a negative control.

The fluorescence signals were measured using a SpectraMax M5 microplate reader (Molecular devices, USA) in the bottom-reading mode at excitation and emission wavelengths of 485 nm and 535 nm. Fluorescent units of cells were treated with sample (FU_T) and untreated cells (FU_C), as per the following equation;

$$\% \text{ Inhibition} = [1-(FU_T/FU_C)] \times 100 \qquad\qquad (1)$$

The lowest concentration of isolated compound that inhibited bacteria growth by 90 % was reported as the minimum inhibitory concentration (MIC).

Assay of cytotoxic activity of compounds isolated from *P. retrofractum* Vahl against lung cancer cells (SCLC-H22 and NCI-H187) and human gingival fibroblasts (HGF)

The cytotoxic activity of piperine, methyl piperate, sylvatine, and piperlonguminine was determined using the human small cell lung carcinoma (SCLC-H22) and the MTT (3-(4,5-dimethylthizolyl-2)-2,5 diphynyltetrazolium bromide) assay [17]. The cells were cultured in RPMI-1640 medium supplemented with 0.1 % sodium bicarbonate and 2 mM glutamine, penicillin G 200 units/mL, streptomycin 100 µg/mL, and 10 % fetal bovine serum.

The cytotoxic activity against human gingival fibroblasts (HGF) was done by Sulforhodamine B (SRB) assay [18]. Normal human cells (HGF) were cultured in complete medium composed of DMEM (Dulbecco's Modified Eagle Medium) 13.5 g, with sterile water 1,000 mL, $NaHCO_3$ 3.7 g, and antibiotic-antimycotic (50 IU/mL penicillin G sodium, 25 IU/mL streptomycin sulfate and 0.125 µg/mL amphotericin B).

Both cell types were trypsinized using 0.25 % trypsin-EDTA to give a single cell suspension, and were treated with 0.4 % tryphan blue stain, prior to counting the cell numbers using a haemocytometer. Cell suspension (100 µL) was seeded into 96-well plates at a density of 5,000 cells/well and incubated at 37 °C in a humidified atmosphere containing 5 % CO_2 and 80 % water humidity for 24 h. The cells were treated with 100 µL of crude methanol extract and isolated compounds (piperine, methyl piperate,

sylvatine, and piperlonguminine), respectively, in 6 replicates. The concentration of crude extract was 25 and 5 µg/mL for the isolated compounds. The plates were incubated, as described above, for 72 h, the media were removed and replaced with fresh media, and the plates were incubated for a further 72 h.

For the SCLC-H22, MTT reagent (20 µL) was added to each well, and the plates were returned to the incubator for 4 h. Acid-isopropanol (100 µL) was added to each sample well, and the plates were stored at room temperature in the dark for 2 h, followed by centrifuging at 250×g for 3 min. The supernatant was transferred to an empty well in a new plate by pipetting, and the plate was covered and stored in the dark overnight at room temperature. The plate was then measured at 570 nm by a microplate reader.

For the normal human cells, after 72 h, the HGF cell was fixed with 40 % trichloroacetic acid (TCA) and kept at 4 °C for 1 h, then rinsed with water 4 times. Plates were allowed to air-dry at room temperature. After that, 50 µL of 0.4 % sulforhodamine B (SRB) dissolved in 1 % acetic acid was added, and left at room temperature for 30 min, then rinsed with 1 % acetic acid 4 times. The plate was allowed to air-dry at room temperature. Then, 100 µL of 10 mM Tris base (pH 10.5) was added to each well, and shaken in order to disperse the SRB color. The OD was measured at 492 nm by a microplate reader.

The percent cytotoxic activity was calculated using the equations;

$$\% \text{ of cell growth} \quad = \quad \frac{\text{OD sample - OD blank}}{\text{OD control - OD blank}} \times 100 \tag{2}$$

$$\% \text{ inhibition} \quad = \quad 100 - \% \text{ of cell growth} \tag{3}$$

The NCI-H187 cell line (ATCC CRL-5840) was derived from small cell lung carcinoma. Cells were cultured in complete medium (RPMI-1640), supplemented with 15 % heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 2.5 g/L glucose, and 2.2 g/L sodium bicarbonate. Cells were maintained at 37 ° C in a humidified incubator with 5 % CO_2 atmosphere, and were harvested in the logarithmic phase of growth. The cell suspension was diluted to 6.7×10^4 cells/mL using complete medium prior to testing. This assay was performed in a 384 well plate in triplicate using the resazurin microplate assay (REMA) [19]. Samples were solubilized with 100 % DMSO and diluted to 0.5 mg/mL with sterilized water, followed by a 3-fold serial dilution with 5 % DMSO. Each well had 5 µL of test compound and 45 µL of cell suspension. The plates were then incubated at 37 °C in a humidified incubator with 5 % CO_2 for 5 days. After that, 12.5 µL of 0.0625 mg/ml resazurin solution was added to each well, and the plates were further incubated at 37 ° C for 4 h. Fluorescence was measured at 530 nm excitation and 590 nm emission wavelength by using the bottom reading mode of the fluorometer. The blank signal was subtracted for the calculation. The percentage of growth inhibition was calculated by the following equation;

$$\% \text{ Inhibition} = [1-(FU_T/FU_C)] \times 100 \tag{4}$$

where FU_T and FU_C represent the fluorescence units of cells treated with test compound and those treated with 0.5 % DMSO, respectively.

The IC_{50} value was derived from the dose response curve that was plotted between % inhibition against the sample concentrations using SOFTMax Pro software (Molecular Devices, USA)

Results and discussion

Antibacterial activity of compounds isolated from *P. retrofractum* Vahl against *M. tuberculosis*

The antibacterial activity of compounds isolated from *P. retrofractum* Vahl (**Figure 1**) against *M. tuberculosis* is shown in **Table 1**. Only piperine and piperlonguminine were found to be active, with a relative high MIC value of 50 µg/mL. Methyl piperate and sylvatine were considered to be inactive, since

less than 90 % inhibition was measured at a concentration of 50.00 µg/mL. Piperine isolated from the fruits of *P. chaba* has previously been reported to display activity against *M. tuberculosis*, with an MIC of 50.00 µg/mL using the MABA assay [20]. However this is the first report of piperlonguminine's activity against the same bacteria, and the mechanism of the alkaloids is not yet clear. The activity of various alkaloids (adhatodine, anisotine, vasicine, vasicinone, vasicolinone, vasicoline) was probed using docking simulation, and considered to involve inhibition of the enzyme β-ketoacyl-acyl-carrier protein synthase III, which is important for the biosynthesis of the fatty acid that is a vital component of the cell wall [21].

In the presented study, short chain alkylated alkaloids were found to be more active than longer chain alkylated alkaloids and the crude methanol extract, exhibited the lowest MIC (25.00 µg/mL) suggesting the presence of more potent compounds in *P. retrofractum* Vahl. The high MIC values of 50.00 µg/mL measured for piperine and piperlonguminine indicate their low potential as agents against tuberculosis. Rifampicin in particular is characterized by a very low MIC, in the range of 0.003 - 0.025 µg/mL. However, the combination of piperine and piperlonguminine with conventional drugs may offer benefits in treating infections caused by resistant strains of *M. tuberculosis*. Piperine has been reported to potentiate the activity of rifampicin against rifampcicin-resistant *M. tuberculosis*. The compound has also been reported to inhibit Ra1258c, a multidrug resistant gene [22]. Piperine also showed inhibition against the efflux pump molecule in *M. smegmatis* [23].
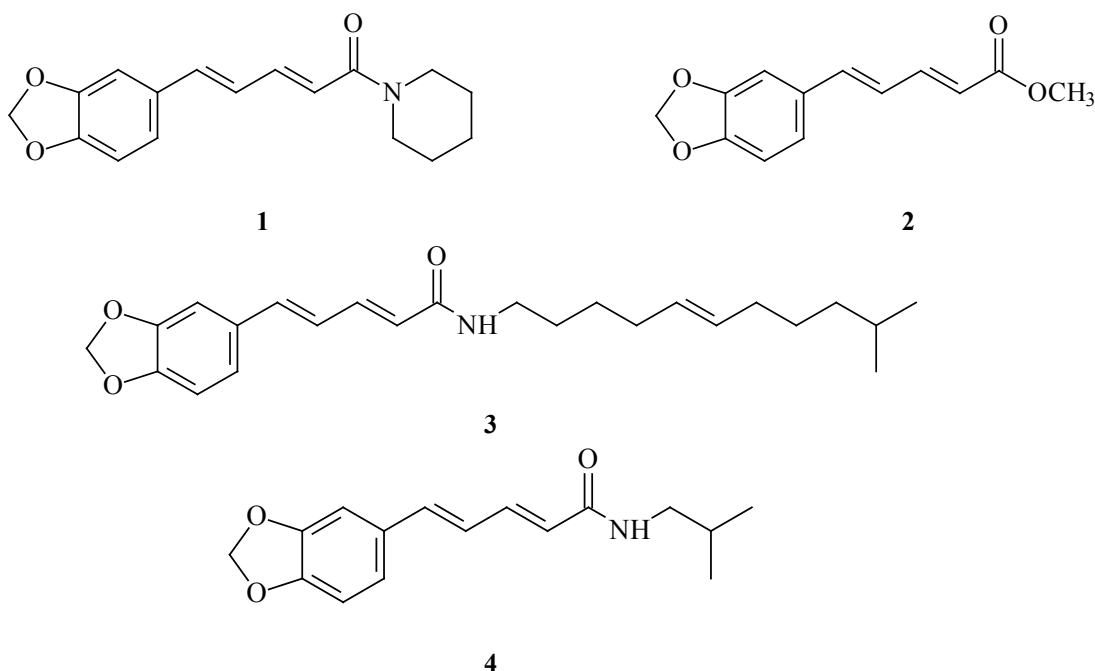

Figure 1 Structure of compounds isolated from *P. retrofractum* Vahl: piperine (1); methyl piperate (2); sylvatine (3); piperlonguminine (4).

Table 1 Anti-*Mycobacterium tuberculosis* (Anti-TB) $H_{37}Ra$ strain of crude methanol extract and isolated compounds.

Compounds	% inhibition [a]	Activity	MIC (µg/mL)
Crude methanol extract	-	Active	25.00
Piperine	119.20	Active	50.00
Methyl piperate	8.59	Inactive	-
Sylvatine	29.58	Inactive	-
Piperlonguminine	107.12	Active	50.00
Isoniazid		Active	0.023 - 0.047
Rifampicin		Active	0.003 - 0.025
Ethambutol		Active	0.234 - 0.469
Ofloxacin		Active	0.391 - 0.781
Streptomycin		Active	0.156 - 0.313

[a] % inhibition at final concentration (50.00 µg/mL)

Active: % Inhibition $\geq$ 90 %

Inactive: % Inhibition < 90 %

Cytotoxic activity of compounds isolated from *P. retrofractum* Vahl against lung cancer cells

All 4 compounds isolated from *P. retrofractum* Vahl showed low cytotoxic activity and poor inhibition of SCLC-H22 lung cancer cell growth (**Table 2**) at a concentration of 5 µg/mL. Sylvatine, piperine and methyl piperate showed increased cytotoxic activity against NCI-H187 lung cancer cells at a concentration of 50 µg/mL compared with SCLC-H22 cells at 5 µg/mL, as expected. Piperlonguminine exhibited similar cytotoxic activity against the 3 cell types investigated, indicating an absence of selectivity. None of the isolated compounds showed potent cytotoxic effects, as demonstrated by a % inhibition of not more than 20 % at a concentration of 5 µg/mL, or a % inhibition of not more than 50 % at a concentration of 50 µg/mL (**Table 2**).

A previous study found that piperine showed some cytotoxic activity against solid tumors in rats [24] and that the compound may confer advantages in combination therapy. Piperine in combination with 5-fluorouracil (5-FU) has been reported to increase the cytotoxic effects of 5-FU on leukemia (HL-60), colon cancer (HCT-8), brain cancer (SF295), and breast cancer (MDA-MB435) cells [25].

The crude methanol extract showed significantly higher cytotoxicity at a concentration of 50 µg/mL against NCI-H187 lung cancer cells (IC_{50} 20.98 µg/mL) compared with the isolated compounds (**Table 2**). This finding suggests a synergistic effect of the mixture of compounds, or the presence of unidentified active compounds in the crude methanol extract. Doxorubicin and ellipticine were used as standard anti-cancer drugs in this study for comparison, and exhibit potent cytotoxic effects against NCI-H187 cells with IC_{50} values of 0.11 and 4.23 µg/mL, respectively. The present findings suggest that the clinical advantages of the crude extract and isolated compounds of *P. retrofractum* Vahl lie in combination therapy in order to enhance the efficacy of conventional anticancer drugs.

Table 2 Cytotoxic activity of compounds isolated from *P. retrofractum* Vahl against lung cancer cells (SCLC- H22 and NCI-H187) and human gingival fibroblasts (HGF) in cell cultures.

Compounds	% inhibition		
	SCLC-H22[*]	HGF[*]	NCI-H187[**]
Crude methanol extract	38.83 ± 1.2	56.83 ± 10.7	96.71 (20.98)[***]
Piperine	7.68 ± 4.8	2.49 ± 8.0	15.58
Methyl piperate	3.67 ± 7.2	0.08 ± 8.3	20.72
Sylvatine	6.60 ± 2.9	20.35 ± 4.0	34.79
Piperlonguminine	11.59 ± 7.5	11.77 ± 1.7	15.04
Ellipticine	-	-	87.98 (4.23)
Doxorubicin	-	-	96.76 (0.11)

[*] SCLC-H22 and HGF cell lines exposed to a concentration of 5 µg/mL for the isolated compounds and 25 µg/mL for the crude methanol extract.
[**] NCI-H187 cell line exposed to a concentration of 50 µg/mL for the isolated compounds and the crude methanol extract.
[***] The number in parenthesis is the IC_{50} value in µg/mL.
Standard drug, ellipticine and doxorubicin, with final concentrations of 8 and 0.8 µg/mL, respectively.

Conclusions

The crude methanol extract from the fruits of *P. retrofractum* exhibited antibacterial activity against *M. tuberculosis* with piperine and piperlonguminine, proving to be the active component, with an MIC value of 50 µg/mL. Methyl piperate, piperine, sylvatine, and piperlonguminine exhibited low cytotoxicity against NCI-H187 lung cancer cells in cultures at a concentration of 50 µg/mL, characterized by growth inhibition of up to 35 %. The crude extract displayed inhibition in excess of 95 % at the same concentration, suggesting the presence of unidentified compounds of increased potency.

Acknowledgements

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