

Analysis of proteins, polysaccharides, glycosaponins contents of *Piper sarmentosum* Roxb. and anti-TB evaluation for bio-enhancing/interaction effects of leaf extracts with Isoniazid (INH)

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Abstract

Piper sarmentosum Roxb. is a tropical plant used as a vegetable and traditional medicine to cure many ailments. Some constituents of the plant have shown antimycobacterial activity. The present study was aimed to investigate the extracts of different parts of the plant for primary metabolic contents, anti-TB evaluation for bioenhancing, interaction effects of leaf extracts with isoniazid (INH) using *Mycobacterium tuberculosis*. For primary metabolic contents, extracts of the plant were evaluated for total proteins, polysaccharides and glycosaponins. Aqueous and ethanol extracts of different parts of the plant have exhibited different contents of total proteins, total polysaccharides and total glycosaponins ($P < 0.01$). Ethanol extracts have shown high contents of proteins and polysaccharides while contents of glycosaponins were high in aqueous extracts. The various extracts of the leaf, petroleum ether, chloroform and methanol exhibited anti-TB activities with MICs at 25, 25 and 12.5 µg/ml, respectively. Ethyl acetate and chloroform fractions of the methanol extract and isoniazid exhibited MICs at 3.12, 3.12 and 0.5 µg/ml, respectively. Isoniazid and both the fractions were combined at a ratio 3: 1, 1: 1 and 1: 3 and evaluated for anti-TB activity. Fractional inhibitory concentration indexes (FICIs) of the combinations were < 0.5 and < 4 . FICI of 1: 1 combination of isoniazid and ethyl acetate fraction was 0.58, which shows that ethyl acetate fraction and isoniazid possess some synergy but it is not statistically significant. The study indicates the nutritional properties, anti-TB and some bioenhancing properties of the plant.

Keywords: Anti-TB, Bio-enhancing, Anti-TB drug, Isoniazid, INH, *Piper sarmentosum*, Proteins, Polysaccharides, Glycosaponins, *Mycobacterium tuberculosis*.

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Piper sarmentosum

cough, flue, pleurisy and lumbago. Being a potential candidate as a traditional medicine, plant has been investigated for a number of activities like anti-amoebic⁴, antibacterial⁵, anti-neoplastic⁶, neuromuscular blocking⁷, hypoglycaemic⁸ and anti-malarial⁹. Leaf extracts are reported to have antioxidant properties due to flavonoids^{10, 11}. Fruit of the plant has shown anti-TB activity and different bioactive amides have been isolated¹².

Tuberculosis (TB) is an intracellular infectious disease caused by different species of *Mycobacterium*,

Introduction

Piper sarmentosum Roxb. (Family: Piperaceae) is cultivated and found as wild in India and South East Asian region. Plant is herbaceous, creeping or erect. It has tender, bright green, ovate to sub orbicular leaves with distinct veins. Male plant spikes white 1.5-2.5 cm × 2-3 mm while female spikes 2-5 cm long and fruit 8 mm. It is available round the year. Phytochemically the plant contains

constituents like alkaloids (amides), pyrones, flavonoids, sterols and neolignans¹⁻³.

The plant is well known due to its culinary and medicinal properties. As a traditional remedy, root and fruit are used to cure dysentery and also combined with table salt to relieve toothache. The whole plant is carminative and given as a drink to relieve symptoms of malaria. Root is believed to be effective in enurea,

mainly *M. tuberculosis*. According to WHO reports, regardless of global efforts and potent therapeutic agents, still TB is more fatal compared to other infections. It has been reported as the largest infectious disease¹³. Isoniazid, rifampicin, ethambutol and pyrazinamide are the most effective oral anti-TB drugs. All these drugs must be taken together during the first 2 months of treatment. Though, adverse reaction are low, many patients experience untoward effects while taking more tablets/capsules together at one time, which is the main cause of non compliance. It also results in resistance and mortality¹⁴. Other main causes of resistance are inconsistent and lengthy treatment, prescription of wrong combinations and sub-standard drugs¹⁵. As new drugs are coming, resistance to existing drugs is increasing¹⁶. Multi drug resistant (MDR) strains are not only difficult to treat but are more fatal also. These complications can be reduced by inclusion of synergistic drugs in the usual treatment. Some combinations have been tried in the treatment of different diseases to, increase efficacy, shorten duration of treatment, improve compliance and reduce risk of resistance¹⁷. Moreover, some vegetables and traditional medicines have anti-TB activity and are being consumed routinely. Therefore, it will be interesting to evaluate food drug interactions. Keeping these in view, it is important to carry out research to find new anti-TB drugs and interaction of anti-TB drugs with commonly used food materials.

Some dietary components and phytochemicals are reported to have bioenhancing and synergistic effect on other drugs¹⁸⁻²³. Grape fruit juice increases

the plasma concentration of some drugs^{24,25}. In Piperaceae, *Piper nigrum* Linn./ piperine has been studied extensively and reported to enhance bioavailability of many drugs by inhibiting several cytochrome P₄₅₀ and phase II reactions²⁶⁻³¹. These bio-enhancers are beneficial to reduce the drug load and improve patient's quality of life.

Natural flora has served human being by giving drugs and supplements. Research in natural products has given a number of drugs and recently a novel compound, isolated from algae was found active against MDR TB³². Considering the potential and biodiversity of natural flora, it is important to explore it for new drug prototypes and bio- enhancers/ interactions.

The aim of present study is to investigate, different extracts of various parts of *P. sarmentosum* for total contents of proteins, polysaccharides, glycosaponins and anti-TB evaluations for bio-enhancing/interactions effects of leaf extracts. So far, there are no reports on these lines. Fruit of the plant is reported to have anti-TB activity, which is due to amides¹². In present study, leaf of the plant has been investigated for anti-TB activity and interaction studies. Leaf was selected because it is mostly consumed as vegetable and phytomedicine.

Materials and Methods

Extraction

Plant material was collected and authenticated. A specimen voucher (0071/06) was deposited in Herbal Secretariat, School of Pharmaceutical Sciences, Universiti Sains Malaysia. Plant was separated into different parts (root, stem,

leaves and fruit) and dried at 40°C. These parts were pulverized and extracted with ethanol and water for one hour by reflux. Ethanol extracts were dried under reduced pressure at 40°C while aqueous extracts were dried in freeze dryer. Leaf powder was also extracted sequentially using petroleum ether, chloroform and methanol. Methanol extract was fractionated with hexane, chloroform, ethyl acetate, *n*-butanol and water.

Chemicals, solvents, bacteria and instrument

Chemicals and solvents were of analytical grade, which include methanol (Merck), ethanol (Merck), water (Universiti Sains Malaysia), Isoniazid (Sigma Aldrich), *Mycobacterium tuberculosis* H37Rev (Public Health Laboratory, Sungai Buloh, Selangor, Malaysia), 96 well microtitre plates, 7H9 broth (Difco Middlebrook), DMSO (Sigma Aldrich), Bovine Serum Albumin Fraction V (Sigma Aldrich), glucose (Sigma Aldrich), *p*-iodonitrotetrazolium (Sigma Aldrich) and Folin- Ciocalteu reagent (Sigma Aldrich). Lambda 45 UV/VIS Spectrometer (Perkin Elmer) instrument was used for the study.

Estimation of total proteins

Total protein estimation was performed by a method described by Lowry *et al*³³. Fifty milligram extract was mixed with 10 ml distilled water in centrifuge tube. After vortex for 2 min, tube was centrifuged for 10 min at 2700 rpm. Supernatant was used for analysis. A volume of 0.1 ml supernatant was taken in a test tube and after making the volume 1ml with distilled water, 3 ml of reagent C were added, which was made by mixing

50 ml of reagent A (2% sodium carbonate in 0.1N sodium hydroxide) and 1 ml of reagent B (0.5% copper sulfate in 1% potassium sodium tartarate). After adding 0.2 ml of Folin- Ciocalteu reagent, tube was incubated for 30 min at room temperature. Bovine Serum Albumin (Fraction V) was used as standard in a range of 12.50 to 100 µg/ml. All the samples and standards were prepared in triplicates and absorbance was measured at 600nm against a blank having all the reagents except the sample. Total proteins were calculated from linear regression equation, obtained from the standard curve.

Estimation of total polysaccharides

Sample (0.2 g) was dissolved in 7 ml hot ethanol (80%) in a centrifuge tube to remove sugars. After vortex for 2 min, tube was centrifuged at 2700 rpm for 10 minutes. With residue, procedure was repeated several time until washing did not give colour with enthrone reagent. The residue, dried on water bath, was extracted with 5 ml of distilled water and 5 ml of 25% HCl at 0°C for 20 minutes. Then tube was centrifuged at 2700 rpm for 10 min and supernatant was saved. Extraction was repeated to get supernatant again. Supernatant was collected in 100 ml volumetric flask and made the volume with distilled water. A volume of 0.1 ml of supernatant was transferred to a test tube and after making the volume 1 ml with distilled water, 4 ml of enthrone reagent were added. Tube was heated for 8 min in boiling water bath, cooled rapidly and intensity of green colour was measured at 630 nm against a blank having all the reagents except sample. Standard solutions of glucose was prepared from 20, 40, 60,

100 and 200 µg/ml and treated in same way. All samples and standards were prepared in triplicates. Concentration of the glucose was calculated from the linear regression equation, obtained from the standard curve. Starch contents were calculated by multiplying the glucose contents obtained from standard curve with factor 0.9.

Estimation of total glycosaponins

One gram extract was dissolved in 50 ml methanol, refluxed for 30 min and filtered. This process was repeated twice. Filtrate was concentrated to 10 ml using rotary evaporator and saponins were precipitated by adding extract drop wise to 50 ml acetone in tarred beaker. Precipitate was dried in oven at 100°C to constant weight and calculation was done by following formula:

$$\text{Glycosaponins} = \left(\frac{\text{Weight of precipitate}}{\text{Weight of sample}} \right) \times 100$$

Anti-TB and interaction studies

Sample preparation for anti-TB studies

Stock solutions of aqueous and ethanol extracts of different parts of the plant, methanol extract of leaf and its chloroform and ethyl acetate fractions were prepared in DMSO (dimethyl sulfoxide) in a concentration of 1 mg/ml. Stock solution of INH was also prepared in DMSO in a concentration of 1 mg/ml. Working solutions of concentration (100 µg/ml) were prepared from the stock solution by further dilution with DMSO.

For interaction studies, working solution of INH was mixed with working solutions of both the fractions in ratios 3: 1 v/v (75 µg/ml: 25 µg/ml), 1: 1 v/v

(50 µg/ml of each) and 1: 3 v/v (25 µg/ml: 75 µg/ml), respectively.

Determination of MIC (MTT assay)

The extract, fractions and INH were tested in a range from 0.195 to 100 µg/ml and MIC was determined by MTT assay³⁴, briefly described as: Sterile deionized water (200 µl) was added to all outer perimeter wells of a sterile 96-well plate and 100 µl of the 7H9 broth were added in wells from rows B to G in columns 3 to 11. Test sample 100µl (100 µg/ml) was added in rows B to G in column 2 and 3. From column 3, 100 µl contents were transferred to column 4 and a similar 1: 2 dilutions were continued to column 10 and 100 µl of excess medium was discarded from the wells of column 10. *Mycobacterium tuberculosis* inoculums (100 µl) were added to the wells in rows B to G in columns 2 to 11. The wells in column 11 served as drug free control. Plates were sealed and incubated at 37°C for 5 days. Tetrazolium-tween 80 mixture (1.5 ml of tetrazolium at a dilution of 1 mg/ml in absolute ethanol and 1.5 ml of 10% tween 80) was prepared and a volume of 50 µl was poured in well B11 and the plate was then incubated at 37°C for further 24 hours. The well B11 was turned purple and tetrazolium-tween 80 was added to all the wells. The plates were sealed and incubated at 37°C and after 24 hours, colour of all wells were recorded. A yellow colour in the well was interpreted as no growth.

Interaction calculations

MIC of the INH, the extract, the fractions and the combinations were determined by serial dilutions as

mentioned in section MIC determination. The interaction was evaluated by standard two dimensional checkerboard assays and interpreted/described previously^{35, 36}. From MIC of the combinations, new MICs of the individual components were calculated. MICs of the alone and MICs in the combination were used to calculate fractional inhibitory concentrations (FICs) and fractional inhibitory concentration indexes (FICIs) using following equations.

$$FIC = [MIC \text{ in combination} / MIC \text{ alone}]$$

$$FICI = \sum FIC \text{ of components}$$

Statistical analysis

All the tests were performed in triplicates and results were averaged. Data was analyzed by using One Way ANOVA using SPSS 12.0.

Results and Discussion

The aqueous and ethanol extracts of different parts of *P. sarmentosum* were investigated for some primary metabolites. Total protein contents were estimated using linear regression equation ($Y = -1.486738e^{03} + 2.310490e^{03}X$, $R^2 = 0.9944$), which was obtained from calibration curve of bovine albumin (fraction v). Total polysaccharides were estimated by linear regression equation ($Y = 1.221024e^{02} + 8.006043e^{04}X$, $R^2 = 0.9984$), which was obtained from the standard curve of glucose. Total glycosaponins were estimated by gravimetric assay. The results of total primary contents are shown in Table 1, which indicate the percentage contents of total contents of primary metabolites and their distribution in various parts of the plant as well as the effect of solvent on

Table 1: Percentage total contents of proteins, polysaccharides and glycosaponins of ethanol and aqueous extracts of different parts of *Piper sarmentosum*

Name of extract		Total protein (%)	SD	Total poly-saccharides %	SD	Total glyco-saponins (%)	SD
Fruit	ethanol	16.84	± 0.01	19.50	± 3.90	30.56	± 0.90
	water	13.58	± 0.65	9.07	± 0.92	40.34	± 0.52
Stem	ethanol	9.84	± 0.01	25.00	± 0.03	27.01	± 0.23
	water	7.97	± 0.15	5.75	± 0.31	47.41	± 0.37
Leaf	ethanol	28.05	± 0.23	21.25	± 0.11	25.03	± 0.21
	water	23.79	± 0.67	9.83	± 0.34	33.10	± 0.24
Root	ethanol	9.12	± 0.43	8.01	± 0.33	20.04	± 0.13
	water	7.13	± 0.37	6.50	± 0.57	26.01	± 0.17

the extraction of these metabolites. The distribution of total metabolic contents was in the order: proteins leaf > fruit > stem > root; polysaccharides stem > leaf > fruit > root; and glycosaponins stem > fruit > leaf > root.

The data was analyzed by one way ANOVA with Tukey's B test. The data within the groups was homogeneous ($P=0.002$). Both the types of extracts of different parts have exhibited different contents of proteins, polysaccharides and glycosaponins ($P>0.01$). The effect of solvent on extraction of total metabolites was also different ($P>0.01$). The contents of proteins and polysaccharides were higher in ethanol extracts as compared to water extracts. In case of glycosaponins, aqueous extracts were higher in contents as compared to ethanol extracts. It means that ethanol is a better solvent for the extraction of total proteins and total polysaccharides while water is better for the extraction of glycosaponins. Polysaccharides and proteins were less soluble in water due to

their structural features.

Sequential extracts of leaf (petroleum ether, chloroform, methanol and water) were investigated for total proteins, polysaccharides and glycosaponins and the results are given in Table 2. The contents of proteins and polysaccharides were high in methanol extract while contents of proteins were not different in chloroform and water extracts. Petroleum ether extract was found to have no contents of these metabolites. The contents of glycosaponins were high in aqueous extracts as compared methanol extract. The high contents of glycosaponins in aqueous extracts were due to their more solubility. The presence of water soluble glycone, more soluble in water, plays an important role in the extraction of glycosaponins.

Sequential extracts of the leaf, petroleum ether, chloroform and methanol, exhibited anti-TB activity with MICs at 25, 25 and 12.5 µg/ml, respectively (Fig. 1). The fractions of the

Table 2 : Percentage total contents of proteins, polysaccharides and glycosaponins in different extracts of the leaf of *Piper sarmentosum* sequential extraction

Name of extract	Total protein (%)	SD	Total poly-saccharides (%)	SD	Total glyco-saponins (%)	SD
Petroleum ether extract	0.00	±0.00	0.0003	±0.01	0.00	±0.00
Chloroform extract	14.02	± 2.15	0.0004	± 0.02	4.64	± 0.12
Methanol extract	25.37	± 3.31	0.0003	± 0.03	30	± 0.16
Water extract	20.74	± 2.01	0.0025	± 0.01	40	± 2.31

methanol extract, hexane, chloroform and ethyl acetate, and INH exhibited anti-TB activity with MIC at 25, 3.12, 3.12 and 0.50 µg/ml, respectively. The chloroform and the ethyl acetate fractions, being more potent, were evaluated for interaction studies. INH and both the fractions were mixed in different proportions and evaluated for anti-TB activity. The combinations of INH and the chloroform fraction, (3: 1), (1: 1) and (1: 3), exhibited MIC at 0.50, 1.6 and 3.12 µg/ml, respectively while the combinations of INH and ethyl acetate fraction, (3: 1), (1: 1) and (1: 3), exhibited MIC at

0.50 µg/ml (Fig. 1). The MICs of the individual components and combinations were used to calculate fractional inhibitory concentrations (FICs) which were then used to calculate fractional inhibitory concentration indexes (FICIs). The results are interpreted as: FICI < 0.5 (synergism), FICI > 0.5 < 4 (no interaction) and FICI < 4 (antagonism). The FICIs of all the combination were less than 0.5, which indicate that there is no interaction. Only combination of INH and ethyl acetate (1: 3) has exhibited FICI at 0.58, which show bio-enhancing to some extent. In this combination, though

concentration of INH was reduced from 100 µg/ml to 25 µg/ml, anti-TB activity was achieved at 0.50 µg/ml.

Conclusion

Aqueous and ethanol extracts of different parts of *P. sarmentosum* contain different contents of total proteins, total polysaccharides and total glycosaponins. Methanol is a better solvent for the extraction of proteins and polysaccharides while water is a better solvent for the extraction of total glycosaponins.

Aqueous and ethanol extracts of different parts of the plant (root, stem, leaves and fruit) are known to possess anti-TB activity with MIC at 12.5 µg/ml. Methanol extract of the leaf obtained by sequential extraction has also exhibited anti-TB activity with MIC at 12.5 µg/ml. Ethyl acetate and chloroform fractions of the methanol extract have shown anti-TB activity with MIC 3.12 µg/ml. There is some enhancement of activity because the combination still achieves MIC at 0.5 µg/ml though the concentration of INH was reduced to 75%.

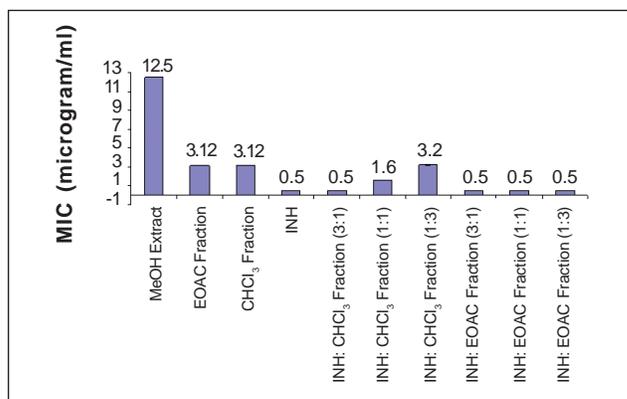


Fig. 1: Anti-TB activity: Minimum Inhibitory Concentration (MIC) in µg/ml of methanol extract of the leaf of *Piper sarmentosum*, chloroform (CHCl₃) and ethyl acetate (EOAC) fractions of the methanol extract, Isoniazid (INH) and different combinations of Isoniazid and the fractions.

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