Journal of Applied Pharmaceutical Science Vol. 8(05), pp 134-138, May, 2018
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2018.8518
ISSN 2231-3354

Synergistic Study on n-Hexane Extract of *Anredera cordifolia* (Ten.)v Steenis (binahong) Leaves Combined with Antituberculosis Drugs against Drug-Sensitive and Drug-Resistant of *Mycobacterium tuberculosis*

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ARTICLE INFO

Article history:
Received on: 16/12/2017
Accepted on: 25/03/2018
Available online: 30/05/2018

Key words:
Anredera cordifolia, *Mycobacterium tuberculosis*, Drug combination, Susceptibility test.

ABSTRACT

Objectives: The aim of this study was to evaluate the antimycobacterial activity of *Anredera cordifolia* (Ten)v Steenis (binahong) leaves extract when it was combined with antituberculosis drugs against drug sensitive and drug resistant of *Mycobacterium tuberculosis* (MTB). *Materials and Methods*: H37Rv sensitive strain, streptomycin-rifampicin (SR) resistant strain, and isoniazid-ethambutol (HE) resistant strain were evaluated by susceptibility test by using a serial number of three different extracts of binahong with the range of concentration was 50 µg/mL-1000 µg/mL. Minimum Inhibition Concentration (MIC) was read as a minimum concentration of extracts that completely inhibited visible growth of the organisms. Synergistic study of extract with anti-tuberculosis (TB) drugs was determined by susceptibility test in Lowenstein Jensen (LJ) media by calculating the Fractional Inhibitory Concentration Index (FICI). *Results*: The results show that n-hexane extract of binahong leaves had better antimycobacterial activity compared to other extracts with the MIC was 500 µg/mL against H37Rv strain or SR strain of MTB and 250 µg/mL against HE strain. The combination of n-hexane extract and anti-TB drugs also displayed a synergistic interaction and no antagonism result from the combination was observed. *Conclusion*: These results indicated that n-hexane extract of binahong leaves may serve as a template for the development of novel antimycobacterial compounds.

INTRODUCTION

TB is the world's second most common causes of death by infectious disease (Ducati *et al.*, 2006). It has been estimated that one third of the world's population is infected by MTB and 10% of these people will become active patients during their lifetime (Bishai *et al.*, 2010). The situation is getting worse day by day owing to the emergence of Multidrug-Resistant (MDR) TB strains and its association with Human Immunodeficiency Virus (HIV) or Acquired Immunodeficiency Syndrome (AIDS) renders its control more difficult. In 2016, 4800.000 people who have MDR-TB increased in the world and 1.9% of TB cases with MDR-TB in Indonesia (WHO, 2016). Thus, there is an urgent need to discover new anti-TB agents that are effective in TB treatment, not only as new synthetic drugs but also natural products from medicinal plants the potential sources of new antimycobacterial agents.

Anredera cordifolia (Ten.)v Steenis is a medicinal plant that is originated from China which is known as Dhen San Chi or Madeira vine in South Africa. In Indonesia, this plant is known as binahong. It is traditionally used to treat various diseases, such as hypertension, gout, and skin disease. Binahong is shown to have anti-inflammatory properties as the hematoma (Sumartiningsih, 2011) hepatoprotective (Lin *et al.*, 1994), a relaxant on the gastric mucosa, anti-obesity, and hypolipidemia (Lin *et al.*, 1997). Binahong leaves juice is also reported to has antimicrobial activity on *Staphylococcus aureus* and *Escherichia coli* with positive control of oxytetracycline (Darsana *et al.*, 2012).

It is reported that the chemical contents of binahong leaves are Ursolic Acid (UA), Oleanolic Acid (OA), and Apigenin. UA and OA have structure of pentacyclic triterpenoid which has

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low solubility in water and high lipophilicity (Codruta *et al.*, 2014). Research conducted by Gracia *et al.* (2015) shown that OA caused a decrease in the growth and development of MTB in macrophages, stimulated the production of nitric oxide in the early phase, and suppressed TGF-α and TGF-β. In addition, dichloromethane extract *Duroia macrophylla* leaves which contained UA and OA showed a significant reduction of activity in rats induced by MTB H37Rv sensitive strain and MDR strain (Daiane *et al.*, 2013; Gayatri *et al.* 2010). A Preliminary study conducted in three different extracts of binahong leaves (n-hexane extract, ethyl acetate extract, and ethanolic extract) showed n-hexane extract had a better anti-TB activity to other extracts of binahong leaves. Meanwhile, synergistic interaction of n-hexane extract combination anti-TB drugs is still unclear.

This study investigated *in vitro* antimycobacterial activity of three different extract of binahong leaves against three different strains of MTB (H37Rv sensitive strain, SR resistant strain, and HE resistant strain) when they were used as individual and combination with first-line antituberculosis drugs. MIC and FICI values were used in order to assess the synergistic activity of n-hexane extract of binahong leaves.

MATERIALS AND METHODS

Plant materials

The sample of Binahong leaves was collected from Manoko, Lembang, West Java. The sample collection was conducted from February to March 2016. It was analyzed at Herbarium Bandungense, School of Life Science and Technology, Institut Teknologi Bandung. The sample was dried in the shade for 10 days. Dried Binahong leaves were extracted using n-hexane, ethyl acetate, and ethanol (1:7) three times repeatedly using reflux method. The solvent was evaporated using a rotary evaporator. Both crude material and extracts were subjected to phytochemical investigation. Qualitative screening included alkaloid, flavonoid, saponin, quinone, tannin, steroid/triterpenoid, and monoterpenoid/sesquiterpenoid.

Microorganism

Antimycobacterial activity was evaluated using MTB H37Rv sensitive strain, SR resistant strain, and HE resistant strain. All bacterial strains were provided from Laboratory of Health Development, West Java Province. Bacterial strains were maintained by subculture on Ogawa 3% for 3 weeks at 37°C for their favorable growth.

Reagents and antibiotics

Antibiotics Rifampicin (RMP), Isoniazid (INH), Streptomycin (STR), and Ethambutol (EMB) were purchased from Sigma Aldrich for combination study and drug control. INH, STR, and EMB were prepared in deionized water and RMP was prepared in Dimethylformamide (Merck). Dimethyl sulfoxide (DMSO) (Merck) was used as a solvent for extracts with the concentration of 0.5 % v/v (De Logu *et al.*, 2000).

In vitro antimycobacterial activity

The antimycobacterial activity of n-hexane extract, ethyl acetate extract, and ethanolic extract of binahong leaves against three different types of MTB was tested by susceptibility test using proportion method in LJ media. The inoculum was adjusted to 3×10^6 CFU/mL by comparison with Mc. Farland no.1 turbidity standard and 100 µl bacteria suspension contained approximately 3×10^5 CFU/mL were spotted onto LJ media in Mc Cartney tubes. Cultivation was done at 37°C for 8 weeks and MICs was read as minimum concentrations of extracts that completely inhibited visible growth of the organisms.

Drugs combination

The extract with the best activity to inhibit the growth of MTB was combined with anti-TB drugs (STR, RMP, INH, or EMB) and determined as described in the determination of MICs. Clearly, 0.125, 0.25, and 0.5 of MIC extract and anti-TB drugs were combined and tested in Lowenstein-Jensen (LJ) media by using susceptibility test. Interpretation of data was achieved by calculating the FICIs as described by De Logu *et al.* (2000):

$$FICI = (MIC_{A\ combination})/(MIC_{A\ alone}) + (MIC_{B\ combination})/(MIC_{B\ alone})$$

The results of data interpretation of FICI are as follows: FICI $\leq$ 0.5, synergies; FICI 0.5-4, no interaction; FICI > 4.0; antagonism.

RESULT AND DISCUSSION

Lots of natural resources are used empirically and have been researched to analyze the alternative agents for treating many diseases. They may contain active compounds which are able to investigate further drug development (Gangwar *et al.*, 2010).

Binahong leaves were collected from one area to minimize the variation of metabolites due to the location. There are several factors which affect metabolites contained in plants such as location, altitude, climate change, and temperature. Metabolites concentration, particularly secondary metabolites, seems to contribute to the pharmacological effect (Gairola *et al.*, 2010).

Phytochemical screening

The aim of phytochemical screening is to identify the secondary metabolites or phytochemical compound that is found in plants. Some of the secondary metabolites that can be derived from plants are alkaloids, flavonoids, saponins, polyphenol, tannins, and terpenoid. Phytochemical screening showed dried leaves of binahong contained alkaloids, terpenoid, quinolones, saponins, and polyphenol. The result of phytochemical screening in crude leaves and extracts of binahong leaves are presented in Table 1.

Several studies showed that pentacyclic triterpenoids are responsible for the antimycobacterial activity. The high lipophilicity of pentacyclic triterpenoids is probably the main factor that allows their penetration through the mycobacterial cell wall (Ge *et al.*, 2010; Joseph *et al.*, 2010).

Three different extract of binahong leaves were screened for antimycobacterial activity by using susceptibility test in drug-sensitive and drug-resistant of MTB. The growth inhibition and MIC values of all the extracts in different strains of MTB are shown in Figure 1. According to that data, it can be shown that n-hexane extract of binahong leaves can completely inhibit the growth of MTB on the concentration of 250-500 µg/mL. N-hexane extract

of binahong leaves showed significant antibacterial activity at MIC value of 500 µg/mL for H37Rv sensitive strain or RS strain and 250 µg/mL for HE strain. DMSO was used as an appropriate solvent control with the concentration of 0.5% v/v which not inhibited the growth of bacteria (De Logu *et al.*, 2000).

Table 1: Phytochemical screening of dried leaves and extracts of binahong leaves.

Parameters	Dried leaves	n-hexane extract	Ethyl acetate extract	Ethanolic extract
Yield (%w/w)	−	1.5	3.20	6.90
Flavonoid	+	−	+	+
Saponins	+	−	+	+
Alkaloids	+	−	−	+
Polyphenol	+	+	+	+
Tannin	+	+	+	+
Quinone	+	+	+	+
Steroid/Triterpenes	+	+	−	−
Mono/Sesquitepenes	+	+	+	+

+ : means detectable; −: undetectable.

Combination testing

Up to now, the treatment for TB needs three to five different drugs simultaneously, depending on the patient's category. Fixed-dose combination (FDC) formulations are currently recommended for the treatment of active TB, Total treatment period was 6 months. Patients first received primary of anti-TB drugs including a combination of 3 or 4 drugs (INH, RMP, EMB, or pyrazinamide (PZA)) during an initial phase of 2 months, followed by a continuation phase of 4 months consisting of INH and RMP (Gulbay *et al.*, 2006).

Each drug of anti-TB has a major role in the killing of MTB. INH and RMP are two major drugs which are used in the treatment of TB. INH is a prodrug and must be activated by catalase-peroxidase enzyme KatG. The activation of INH will inhibit the formation of mycolic acids of the bacterial cell wall causing the death of the bacillus (Zhang, 2005). RMP interferes with transcription by the DNA-dependent RNA polymerase. RMP will bind to the β-subunit hindering transcription and kill the organism (Herrera *et al.*, 2003).

EMB is the first-line of drug that is used the combination of INH and RMP. EMB is bacteriostatic that interferes biosynthesis of cell wall arabinogalactan (Takayama *et al.*, 1989). STR is an aminoglycoside that kills the actively growing tubercle bacilli. STR inhibits protein synthesis by binding to the 30S subunit of the bacterial ribosome, causing misreading of the ribosomal protein (Davies *et al.*, 1965).

The MICs for anti-TB drugs used in this combination study were STR 4 µg/mL, INH 0,2 µg/mL, RMP 40 µg/mL, and EMB 2 µg/mL. Combination of extract and first-line drugs of anti-TB majority showed completed growth inhibition of MTB both sensitive strain and resistant strain of MTB. No interaction was found when the extract was combined with RMP compared to RS strain but the synergistic effect was shown when the extract was combined with STR, INH, EMB, or all first-line drugs compared to RS strain. No interaction result was found when the extract was combined with EMB compared to HE strain, but the synergistic interaction was also shown as the result of the combined extract with STR, INH, RMP, and all first-line drugs. All of the combinations showed the synergistic effect when they were used in H37Rv sensitive strain.

The MICs of n-hexane extract was significantly decreased compared to n-hexane extract when strain are significantly decreased, ranging from 62.5-250 µg/mL, and also the MICs of anti-TB drugs respectively. The FICI values were showed ranging from 0.25-0.5 and some of the combinations were found having FICI values as 1. Noticeably, no antagonism result from the combination of extract with anti-TB drugs observed for any of the strains tested. The result of combination tested is shown in Table 2.

Table 2: Combination testing of n-hexane extract and anti-TB drugs compared to drug sensitive and drug resistant to MTB.

Drug combination	Status of strains	Combination MICs (µg/mL)	FICIs	Conclusion
Extr/STRP	$H37Rv^S$	62.5/0.5	0.25	Sy
	RS^R	125/1	0.5	Sy
	HE^R	62.5/1	0.5	Sy
Extr/INH	$H37Rv^S$	62.5/0.025	0.25	Sy
	RS^R	62.5/0.025	0.25	Sy
	HE^R	125/0.05	0.5	N
Extr/RMP	$H37Rv^S$	62.5/5	0.25	Sy
	RS^R	250/20	1	N
	HE^R	31.25/5	0.25	Sy
Extr/EMB	$H37Rv^S$	125/0.5	0.5	Sy
	RS^R	62.5/0.25	0.25	Sy
	HE^R	125/1	1	N
Extr/STRP/INH/RMP/EMB	$H37Rv^S$	62.5/0.5/0.025/5/0.25	0.25	Sy
	RS^R	62.5/0.5/0.025/5/0.25	0.25	Sy
	HER	31.25/0.5/0.025/5/0.25	0.25	Sy

Extr, Extract; S, Sensitive; R, resistant; RS, rifampicin-streptomycin; HE, isoniazid-Ethambutol; N, no interaction; Sy, synergistic.

The highest activity of the n-hexane extract from binahong leaves in this study could be attributed to the presence of UA and OA as pentacyclic triterpenes. These two compounds are widely distributed in the plant world and are known to have a wide spectrum of activity. The high lipophilicity of triterpenes is probably the main factor that allows their penetration through the mycobacterial cell wall (Wachter *et al.*, 1999). It is showed that triterpenes UA and OA is relatively non-toxic. The mechanism of antimicrobial action of UA and OA has been investigated and was utilized to be the basis on the inhibition of DNA polymerase (Joseph and Priya, 2010). Acids from UA affected cell morphology and enhanced autolysis of the bacterial cells. UA also could inhibit peptidoglycan turnover and could affect the profile of muropeptides after digestion of peptidoglycan with mutanolysin (Kurek *et al.*, 2010). Literature data also reported that OA has a synergistic effect when it is combined with INH, RMP, or EMB (Deng *et al.*, 2008).

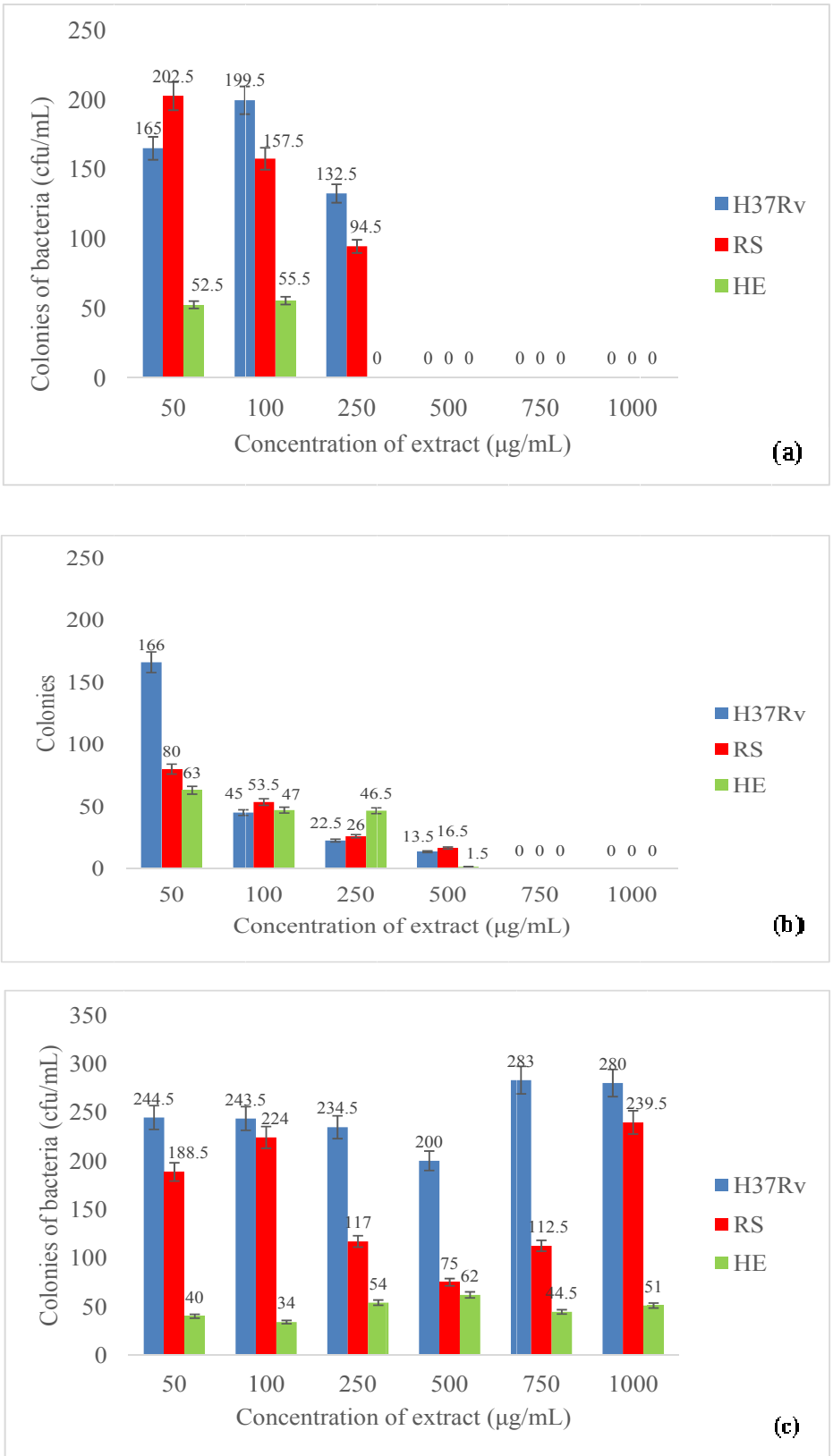

Fig. 1: MTB growth inhibition and MICs of three different extracts (n-hexane extract (**a**); ethyl acetate extract (**b**); and ethanolic extract (**c**)) of binahong leaves against drug-sensitive and drug-resistant strains of MTB.

CONCLUSION

Regarding results of the antimycobacterial activity, n-hexane extract of binahong leaves has the best activity compared to other extracts of binahong. Though, it has been reported that UA and OA that are found in extract and confirmed with phytochemical screening that the extract contained pentacyclic triterpenoid. We, therefore, conclude that these compounds may play a crucial role in the antimycobacterial activity of n-hexane extract of binahong leaves. This is a preliminary result but sufficiently interesting for the further study of n-hexane extract of binahong leaves as a template of antitubercular development.

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How to cite this article:
Pitaloka DAE, Sukandar EY. Synergistic Study on n-Hexane Extract of *Anredera cordifolia* (Ten.)v Steenis (binahong) Leaves Combined with Antituberculosis Drugs against Drug-Sensitive and Drug-Resistant of *Mycobacterium tuberculosis*. J App Pharm Sci, 2018; 8(05): 134-138.