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INHIBITIVE ENHANCEMENT OF ISONIASID TREATMENT ON MYCOBACTERIUM TUBERCULOSIS THROUGH TRITERPENOID CARBOCYLIC ACID FROM RED ALGAE EUCHEMA SPINOSUM

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ABSTRACT

Anti-mycobacterial bioactivity of non polar compounds from red algae *Euchema spinosum* of Barang Lompo Island was studied. A triterpenoid carbocylic acid has been isolated from the chloroform fraction of *Euchema spinosum,* and its structure was determined by spectroscopic evidences including IR and 2D NMR and compared to previous data. The isolate was active against *Mycobacterium tuberculosis* at concentration 4 µg/mL, and could also increase the sensitivity of this pathogen to isoniasid drug. These studies suggest that anti-mycobacterial triterpenoid carbocylic acid in combination with isoniasid may play an important role in host defence against *M. tuberculosis* strain H37Rv.

KEYWORDS:Red algae, *Euchema spinosum*, Triterpenoid carbocylic acid, Antimycobacterial activity.

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INTRODUCTION

Tuberculosis is a chronic infectious disease and is one of the major enemies of humanity from times immemorial. Today it still remains as one of the most serious of medical and social problems. According to estimation by the World Health Organization (WHO), approximately one third of the world's population is infected with *Mycobacterium tuberculosis*, eight million people develop tuberculosis disease annually, while two million people die and another three million new cases are being added each year[1] (WHO, 2008). The advances in the chemotherapy of tuberculosis in the last-20th century have recently given way to anxiety over the evolution of drug resistance based on the genetically fixed mutations of *M. tuberculosis*. Moreover, nearly all drugs used for the treatment of tuberculosis and possessing different mechanisms of activity are able to cause adverse side effects on humans[2]. Therefore, it is extremely important to search for new, low-toxic substances superior to the available drugs in their activity and efficiency. This primarily concerns agents possessing activity against *M. tuberculosis* strains with multidrug resistance.Modern tuberculosis is generally associated with *M. tuberculosis* and *M. bovis*, mycobacteria that are pathogenic to humans. Because of slow growth and pathogenicity of *M. tuberculosis* H37Rv, many research groups use fast-growing and/or nonpathogenic mycobacteria including *M. tuberculosis* H37Ra, *M. smegmatis*, *M. aurum* and others as test organisms. A group of research works includes investigations on *M. tuberculosis* clinical isolates and strains possessing multidrug resistance. Multidrug-resistant tuberculosis (MDRTB) is strictly defined as *M. tuberculosis* strains showing resistance simultaneously against isoniazid and rifampicin[3,4]. Tuberculosis with a different drug resistance (DDRTB) involves *M. tuberculosis* strains displaying mono- or polyresistance not including associated resistance against isoniazid and rifampicin[5]. Very recently, patterns of resistance to commonly used anti-tuberculosis drugs among *M. tuberculosis* complex isolates from patients attending government urban TB diagnostic in Makassar, the capital of the South Sulawesi Province in Indonesia, was found to be highly potential risk factors for MDR-TB[6]. In order to tackle these new situations, it is necessary and important to develop not only new treatment guidelines, such as combination treatment between clinical drug with natural product compounds, but also new anti-mycobacterial drugs for efficacious clinical control of TB patients. On the other hand, the ocean environment contains over 80% of world's plant and animal species[7] and with more than 150,000 seaweeds found in the intertidal zones and tropical waters of the oceans, it is a primary source of natural products[8]. Research in natural products has given a number of drugs and recently a novel compound, isolated from marine algae was found to be active against MDR TB[9,10]. Considering the potential and biodiversity of natural flora in Indonesia especially marine algae, it is important to explore it for new drug prototypes for tuberculosis diseases. In this work, we report for the first time the isolation, purification, and structural determination of triterpenoid carbocylic acid from red algae *Euchema spinosum.* We also tested the activity of triterpenoid carbocylic acid alone and in combination with the anti-tuberculosis drug isoniazid (INH) against *M. tuberculosis* strain H37Rv (ATCC 27294) to increase sensitivity to anti-tuberculosis drugs.

MATERIALS AND METHODS

Materials

The red algae *Euchema spinosum* was collected in the region of Barang Lompo Island, South Sulawesi Province, Indonesian terrestrial. Algae identification was conducted in Marine Biology Laboratory Faculty of Natural Sciences Hasanuddin University, Indonesia. Chloroform, isoniazid, and methanol were purchased from Sigma.

Extraction and Isolation

Dry powder of 3.2 kg of algae was macerated with MeOH for 72 h, filtered, and solvent evaporated to obtain a dark brown extract amounting 312 g. The extracts were partitioned with chloroform resulting in 51 g product. The chloroform extract was fractionated by VLC and produced nine major fractions (I-IX). White precipitate found in the third fraction was filtered by filtration resulting in 49 mg product. Purity test was performed by TLC analysis using three solvent systems and its melting point determined.

Instrumentation

The melting point was determined using a micro melting point measurement (John Fisher, Germany). IR spectra determination was done with a Shimadzu spectrometer (Japan). NMR spectra of ^{1}H, ^{13}C and HMBC were obtained using a Bruker, Germany DPX-500 spectrometer at 300 MHz (^{1}H) and 125 MHz (^{13}C) with TMS as an external standard. Separations and identification of compound were conducted with VLC by Merck Si gel 60 (230-400 mesh), and TLC on aluminum or glass plates coated with Merck Si gel 60 F254 and thickness of 0.25 mm.

Anti-mycobacterial activity assays

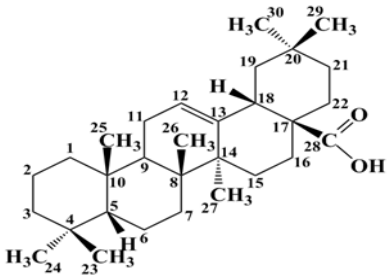
For the initial bioactivity screening, standard colony assays on Löwenstein-Jensen (LJ) medium were performed to assess the anti-mycobacterial activity of Methanol whole extract, chloroform fraction, and compound 1 with the final concentrations of 10 µg/mL, respectively, using the strain *M. tuberculosis* H37Rv (ATCC 27294) and solvent DMSO as negative control plus INH as positive control[11]. For testing the anti-mycobacterial activity of compound 1 and in combination with isoniasid, the strain *M. tuberculosis* H37Rv (ATCC 27294) were grown on Middlebrook 7H19-OADC (oleic acid, albumin, dextrose, catalase) (Difco Laboratories, USA) at 37 °C for 3 weeks until midlog phase. The turbidity of culture were adjusted with 7H9 broth to a 0.5 McFarland standard using a nephelometer, and cell cultures was inoculated on 4 mL MGIT medium[12], using solvent (DMSO) as negative control, isoniasid (0.5 and 1.0 µg/mL), and combination with compound 1 were indicated. Tubes were incubated at 37 °C and inhibition growth documented after 3 days; MGIT tubes were removed from the incubator and placed on a 365 nm UV transilluminator (micro MGIT reader). It is important to read the tube every day beginning on day 3, until results can be interpreted. The growth control tube was compared to the positive and negative controls; positivity was indicated by bright orange fluorescence on the bottom of the tube and an orange reflection at the meniscus; negative tubes on the other hand showed low level or no fluorescence. Once the MGIT growth control is positive, the drug-containing tubes will be read for interpreting results. The drug-containing tubes are interpreted on the same day the MGIT GC is positive and for up two additional day, not to exceed fourteen days. If the MGIT GC tube is not positive by the twelfth day of the test, the test is invalid. Interpret the MGIT result as Susceptible if the drug-containing tube does not fluoresce within two days of onset of fluorescence in the GC tube. Interpret the MGIT result as resistant if the drug-containing tube fluoresces on or within two days of the day of onset of fluorescence in the GC tube. When interpreting resistance, finalize the result as soon as the MGIT GC and the drug-containing tubes fluoresce. All inoculated MGIT tubes after 56 days or negative results should be autoclaved prior to disposal. For positive MGIT media results should add disinfectant (5 % Vesphene) before put in the autoclave bag. MGIT tubes are kept in the autoclave bag place in the autoclave. After finishing autoclaving, send them for the incineration. The growth control tube was compared to the positive and negative controls; positivity was indicated by bright orange fluorescence on the bottom of the tube and an orange reflection at the meniscus; negative tubes on the other hand showed low level or no fluorescence. The assays was conducted in duplicates and repeated three times to produce representative experimental data.

RESULTS AND DISCUSSION

The methanol extract of the dried and powdered form of red algae *Euchema spinosum* was partitioned with chloroform. The chloroform fraction was further separated by silica gel chromatography and crystallized to obtain compound 1. The compound obtained, compound 1 is a white powder crystal, mp 176-177 ^{o}C, giving an indication of triterpenoid on Liebermann-Burchard test. Its IR spectrum showed the absorption bands for hydroxyl (1097), aliphatic groups (2962, 2918 and 2850), C=C (1635), CH_2 (1459), CH_3 (1378), C=O (1705, 1072) and while strong absorption band at 1026 were characterized as a carbocylic acid compound[11].

Table 1
^{1}H (300 MHz) and ^{13}C (125 MHz) NMR data of compound 1 in DMSO (TMS)

Carbon Position	Carbon Type	δc	δH (multiplicity, J (Hz))
1	CH2	70.2	3.43 brs
2	CH2	32.4	1.63 m, 1.82 m
3	CH2	65.1	4.03 dd 13.1, 4.4
4	C	42.0	
5	CH	40.1	1.67 m
6	CH2	17.8	1.13 m
7	CH2	32.1	1.48 m; 1.17 m
8	C	40.2	
9	CH	38.4	2.41 dd 8.0, 8.1
10	C	41.2	
11	CH2	23.5	1.82-1.89 m
12	CH	123.2	1.78 dd 4.1, 8.2
13	C	143.7	5.13 s
14	C	42.4	
15	CH2	26.2	
16	CH2	27.1	2.04 m
17	C	32.3	
18	CH	46.2	2.00 brd
19	CH2	40.7	1.28 m; 2.17 dd 3.2,13.2
20	C	42.5	
21	CH2	30.1	1.79 m
22	CH2	36.4	1.39 brt, 1.28 dd
23	CH3	69.8	3.43 d, 9.6; 3.14 d, 9.3
24	CH3	11.9	0.67 s
25	CH3	15.8	1.01 s
26	CH3	17.4	1.04 s
27	CH3	26.4	1.28 s
28	COOH	181.3	
29	CH3	28.8	0.89 s
30	CH3	19.8	1.21 s



(1)

Figure 1
Structure of triterpenoid carbocylic acid (1) from red algae Euchema spinosum

The ^{13}C NMR and DEPT spectrum exhibited 30 carbon signals (7×CH_3, 11×CH_2, 4×CH, 8×C including COOH), and the molecular formula of compound 1 was deduced to be $C_{30}H_{46}O_2$. The ^{1}H NMR spectrum (Table 1) observed the presence of seven tertiary methyl groups on saturated carbons at d 1.21(s, 3H), 0.89 (s, 6H), 1.28 (s, 6H), 1.04 (s, 3H), 1.01 (s, 3H), which is due to the axial proton attached to C-4 containing the methyl group, one olefinic proton appeared as a double doublet at d 1.78 (dd, 1H, J=4.1, 8.2Hz). Two olefinic carbons at d 123.2 and 143.7 in the ^{13}C NMR (Table 1) spectrum indicated that compound 1 belongs to the triterpenoid, and the double bond C=C is at C-12 and C-13. The position of the carboxylic group at C-17 was confirmed by the HMBC spectrum (Figure 1). The correlation of C-28 (-COOH) with H2-16, H-18 and H-22 were observed in the HMBC spectrum. Based on the data description above and compared with physico-chemical and spectroscopic data of previously known compound[13], the molecular structure of compound 1 is in Figure 1 with name of triterpenoid carbocylic acid. In the first, antimycobacterial activity of all fractions, including compound 1 at concentrations 10 µg/mL was measured by incubating with the mycobacterial cells on LJ medium. MeOH fraction at concentration 10 µg/mL was able to decrease mycobacterial cells growth, where as chloroform fraction and compound 1 in the same concentration, like INH as positive control with no colony growth of *M. tuberculosis* on LJ medium. However, negative control (DMSO) did not display anti-mycobacterial activity (Figure 2). To confirm this finding and to identify the MIC value of

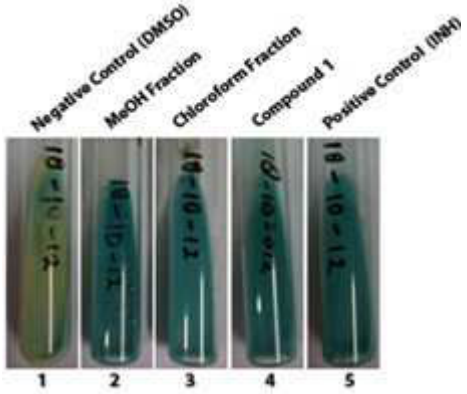


Figure 2
Effect of MeOH whole extract, Chloroform Fraction, and Compound 1 alone on growth inhibition of M. tuberculosis H37Rv (ATCC 27294) strain in Löwenstein-Jensen medium

compound 1, anti-mycobacterial activity of this compound against *M. tuberculosis* test was performed with MGIT medium. The combinations of compound 1 and INH were tested in the anti-mycobacterial assay described above by a checkerboard method in order to identify those combinations that could enhance bioactivity effect. The anti-mycobacterial activity of compound 1 at different concentrations 0, 0.5, 2, and 4 µg/mL in combination with INH at variation concentrations 0, 0.5, 1 µg/mL was measured by incubating the mycobacterial cells on MGIT medium. Compound 1 alone at concentration 0.5 and 2 µg/mL was able to decrease mycobacterial cells growth. Furthermore, at concentration 4 µg/mL showed significant inhibition of growth of *M. tuberculosis* (Figure 3). This result was in accordance with previously reported research by Woldemichael *et al* (2003), where terpenoid compounds from *Calceolaria pinnifolia* (Scrophulariaceae) showed significant inhibition of growth of *M. tuberculosis* with MIC values between 4 and 128 µg/mL[14].

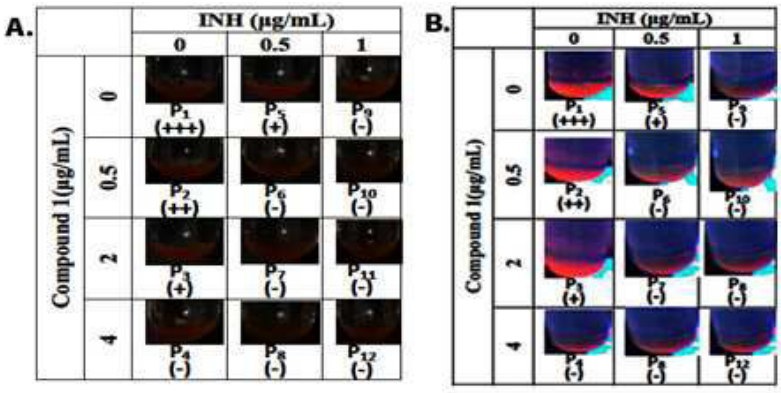


Figure 3

Effect of compound 1 in combination with INH in MGIT medium on growth inhibition of M. tuberculosis H37Rv (ATCC 27294) strain. (A) Before placed on a 365 nm UV transilluminator and (B) after placed on a 365 nm UV transilluminator

In addition, recently reported research by Truong *et al* (2011), that triterpenoid compound from *Radermachera boniana* showed significant inhibition of growth of *M. tuberculosis* with MIC values 4 µg/mL[15]. The combinations with the INH drug at concentration 0.5 and 1 µg/mL could completely inhibit the cells at all concentration of compound 1, although mechanism of anti-mycobacterial action by direct and/or indirect of triterpenoid has not been clear, but it is speculated that membrane disruption of mycobacterial cells by lipophilic compounds involves in the mechanism[16].

CONCLUSION

Compound 1, triterpenoid carbocylic acid was isolated from red algae *Euchema spinosum*. The compound was not only active against *M. tuberculosis* at 4 µg/mL, but also could increase the sensitivity of this pathogen to isoniasid drug. In conclusion, our results demonstrated that the activity isoniasid drug could be enhanced through triterpenoid carbocylic acid. Further studies using *in vivo* methods are required to explore the compound responsible for the activity and the mechanism of this activity which might prove important for improved therapies for the treatment and prevention of tuberculosis.

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REFERENCES

1. World Health Organization (2008). Global Tuberculosis Control: Surveillance, Planning, Financing. WHO report 2008, WHO/HTM/TB/2008.
2. Abdel-Aal, W.S., Hassan, H. Y., Aboul-Fadl T, Youssef, A.F. (2010). *Eur J Med Chem.* 45(3), 1098-1106.
3. Bastian, I., Portaels, F. (2003). In *Multidrug-Resistant Tuberculosis*, Bastian,

I., Portaels, F., Eds., Medicine and life, Moscow, p. 17, 21-22.
4. Mayer, A. M., *et al.,* 2011. Marine pharmacology in 2007–8: *Comp. Biochem. and Physiol.* Part C (153), 191-222.
5. O'Brien, R. J. and Nunn, P. (2001). *Am. J. Respir. Cell Mol. Crit. Car. Med.,* 162, 1055.
6. Massi, M. N., Wahyuni, S., Halik, H., Anita, Yusuf, I., Leong, F. J., Dick, T., and Phyu, S. (2011). *Inter. J. Tuberc. Lung Dis.* 15(4), pp. 489–495.
7. Jha, R.K.; Zi-rong, X. (2004). *Mar. Drugs 2*, 123–146.
8. Copp, B. R. and Pearce, A.N. (2007), *Nat Prod Rep.,* 24(2), 278-297.
9. Prakash, S. and Bhimba, B. V. (2005), *J. Prod. Rad.*, *4*, 264-269.
10. Wachter, G.A., Franzblau, S.G., Montenegro, G., Hoffmann, J.J., Maiese, W. M., Timmermann, B.N. (2001). *J. Nat. Prod.*, *64*, 1463-1464.
11. Yu, X., Jiang, G., Li, H., Zhao, Y,. *et al.* (2011). *J. Clin Microbiol.* 49(3), 784–789.
12. Palomino, J. C., Traore, H., Fissette, K., and Portaels, F. (1999). *Int. J. Tuberc. Lung Dis.* 3(4), 344–348.
13. Mukheree, K. S., Mukhopadhyay, B., Mondal, S., Gorai, D., and Brahmachari, G. (2004). *J. of the Chinese Chem. Society* 51, 229-231.
14. Woldemichael, G.M., Franzblau, S.G., and Zhang, F. (2003). *Planta Med.* 69, 628-631.
15. Truong, N. B., Pham, C. V., Doan H.T. M. *et al*. (2011). *J. Nat. Prod.*, 74 (5), 1318–1322.
16. Termentzi, A., Fokialakis, N., and Skaltsounis, A.L. (2011). *Curr. Pharm. Des.* 17, 1267-1290.