ScienSage

Journal of Advanced Scientific Research

Available online through http://www.sciensage.info/jasr

ISSN
0976-9595
Research Article

Antimycobacterial Sterols from Aromatic Stem Sap of *Commiphora eminii* Engl.

Paul Erasto

Institute of Traditional Medicine, Muhimbili University of Health and Allied Sciences,
P.O. Box 65001, Dar es Salaam, Tanzania.
**Corresponding author: paulkazyoba@yahoo.co.uk*

ABSTRACT

The phytochemical analysis on aromatic stem sap of *Commiphora eminii* afforded two cholesten sterols namely, 4α-methyl-cholest-7-en-3β-ol (**1**) and cholest-7-en-3β-ol (**2**). Their chemical structures were deduced using NMR and MS spectral data and with comparison with data available in literatures. The sterols were screened for antimycobacterial activity against *Mycobacterium madagascariense* (MM) and *Mycobacterium indicus pranii* (MIP). Only sterol **2** exhibited antimycobacterial activity with MIC values of 1.6 mg/ml against both mycobacteria strains used. In the combination assay, sterols potentiated activity of isoniazid (INH) against test organisms, which are known to be resistant to isoniazid even at higher concentration. Sterol **1** had a fractional minimum inhibition concentration (FMIC) of 0.2 mg/ml while sterol **2** had FMIC values of 0.1 against both MM and MIP. The combination of two sterols alone lacked efficacy. The fact that, sterol **1** lacked efficacy in the normal antimycobacterial assay, and that during the combination assay, it potentiated activity of INH at a low concentration indicates that, sterols may be considered as activity enhancers for antibiotics such as isoniazid, especially when testing against drug resistant *Mycobacterium* strains.

Keywords: *Commiphora eminii*, Burseraceae, sterols, isoniazid, *Mycobacterium madagascariense, Mycobacterium indicus pranii.*

1. INTRODUCTION

The genus *Commiphora* (Burseraceae) comprise of over 150 species mainly distributed in East Africa, Arabia and India [1, 2]. The resinous exudates (sap) of *Commiphora* species have for many years been used for various medicinal and cosmetic purposes. The exudates have anti-inflammatory and antimicrobial effects making them useful in the preparation of antiseptics, soaps and deodorants [3- 5].

Commiphora eminii Engl. (J.B. Gillett) is a medicinal plant widely spread in East Africa and used in the treatment of various ailments. In Tanzania, pastoralists use aromatic saps from the stem bark of this species in the treatment of skin infections, gastrointestinal infections and wounds [6], as well as dry and blood cough (unpublished data). Although ethnomedical use of extracts of this species is known, little is known on its phytochemicals and pharmacological properties. Therefore, the claimed ethnomedical uses of this species particularly in the treatment of persistent dry and blood cough has never been investigated. These ailments are among important symptoms of mycobacterial infection in human. Consequently, this paper report the antimycobacterial activity of two cholestene sterols isolated from aromatic stem saps of *Commiphora eminii*. It also report the ability of sterols to potentiate antimycobacterial activity of isoniazid (INH) against INH resistant mycobacteria strains namely, *Mycobacterium madagascariense* and *M. indicus pranii*.

2. MATERIAL AND METHODS

2.1. General analyses

The 1-D and 2-D NMR data of the isolated compounds were obtained using Bruker Avance Ultrashield 400 Plus NMR machine operating at a spectrometer frequency of 400 MHz for ^{1}H-NMR and 75 MHz for ^{13}C-NMR. EI-MS spectra were recorded on a Finnigan MAT SSQ 7000 Single Quadrupole Instrument. Melting points were measured on a Stuart Scientific (SMP1) melting point apparatus.

2.2. Chemicals and Growth Media

All solvents were purchased from Carlo Erba (France), Middlebrook 7H9 broth base was obtained from HIMEDIA (India), Glycerol (AR) obtained from Lab Equip Ltd (Tanzania), iodonitrotetrazolium (INT) chloride, Ciprofloxacin and Isoniazid (R&D) were purchased from Sigma (UK). Ninety six wells micro-titre plates supplied by KAS Medics (Tanzania). Silica gel Kiesegel 60 PF_{254} obtained from Merck South Africa Pty. Pre-coated Aluminium backed silica gel 60 F_{254} (0.2 mm thickness) TLC plates were obtained from Merck UK.

2.3. Plant Materials

The stem bark sap of *Commiphora eminii* was collected from Handeni, Tanga. Authentication of the plant species was done on site by a retired botanist Mr Boniface Mhoro, and a voucher specimen MH 164545 is deposited in the Herbarium at the Institute of Traditional Medicine. The collected saps were stored in a closed glass bottles before being subjected to further phytochemical analysis.

2.4. Liquid-liquid extraction from aromatic sap

Two hundred and seven grams of *Commiphora eminii* saps was mixed with distilled water and homogenised by stirring at 40°C. The sap solution was separately partitioned with dichloromethane, ethyl acetate, thereafter n-butanol to afford 86 g, 40 g and 53 g of extracts respectively. Out of three sets of extracts, only dichloromethane extract showed some antimycobacterial activity with MIC values of 2.5 mg/ml against *Mycobacterium madagascariense*, therefore was picked for further phytochemical analysis.

2.5. Isolation of steroids from dichloromethane extracts

Sixty grams of dichloromethane extract was adsorbed in silica gel 60PF and loaded on a silica gel column eluting with 100% dichloromethane with increasing polarity to 1:1 dichloromethane/acetone. A total of 40 fractions each with 100 ml of eluates were collected. After TLC analysis, fractions 1-15 were discarded as they contained mainly free fatty acids, 16-24 and 25-36 were combines ready for further analysis. The fractions 37-40 were also discarded as it did not have compounds of interest. The combined fractions were rapidly screened for antimycobacterial activity whereby combined fractions 16-24 and 25-36 exhibited some growth inhibition against *Mycobacterium madagascariense.*

The fraction 16-24 was adsorbed in silica gel and loaded on silica gel column eluting with 7.5:2.5 dichloromethane/acetone solvent system. The mobile phase was maintained throughout the elution and 28 sub-fractions each with 50 ml of eluates were collected. After TLC analysis fraction 8 to 28 were combined and left to stand overnight whereby white needle like crystals formed. Crystals were recrystallized in 8:2 Methanol/acetone to give 231 mg of white crystals identified as 4α-methyl-cholest-7-en-3β-ol (**1**).

The remaining fraction 25-36 was adsorbed in silica gel and loaded on silica gel column eluting with 7:3 dichloromethane/acetone solvent system. The mobile phase was adjusted to 6:4 dichloromethane/acetone to afford 18 sub-fractions each with 50 ml of elutes. All sub-fractions were left to stand overnight and white fine needle like crystals weighing 354 mg were collected and identified as Cholest-7-en-3β-ol (**2**).

4α-methyl-cholest-7-en-3β-ol (1): White needle crystals, mp 145-149°C, MS: m/z 400 [100%, M^+], ^{13}C-NMR data (Bruker Avance Ultrashield 400 MHz): δ31.2 (C-1), δ30.9 (C-2), δ76.2 (C-3), δ40.2 (C-4), δ49.9 (C-5), δ28 (C-6), δ117.4 (C-7), δ139.1 (C-8), δ46.6 (C-9), δ43.3 (C-10), δ23.9 (C-11), δ36.2 (C-12), δ37 (C-13), δ56.1 (C-14), δ27.9 (C-16), δ54.9 (C-17), δ14.1 (C-18), δ22.5 (C-19), δ21.3 (C-20), δ18.8 (C-21), δ34.8 (C-22), δ36.1 (C-23), δ29.9 (C-24), δ39.5 (C-25), δ22.8 (C-26), δ15.8 (C-27), δ11.8 (C-28).

Cholest-7-en-3β-ol (2): White crystals, mp 120-125°C, MS: m/z 386 [100%, M^+], ^{13}C-NMR data (Bruker Avance Ultrashield 400 MHz): δ31.4 (C-1), δ29.6 (C-2), δ71 (C-3), δ37.9 (C-4), δ43.3 (C-5), δ21.5 (C-6), δ117.4 (C-7), δ139.6 (C-8), δ49.4 (C-9), δ40.2 (C-10), δ23.9 (C-11), δ36.1 (C-12), δ39.5 (C-13), δ55 (C-14), δ28 (C-15), δ27.9 (C-16), δ56.1 (C-17), δ22.5 (C-18), δ22.9 (C-19), δ18.8 (C-20), δ34.2 (C-21), δ36.2 (C-22), δ22.5 (C-23), δ39.4 (C-24), δ37 (C-25), δ13 (C-26), δ11.8 (C-27).

2.6. Antimycobacterial screening of sterols

2.6.1. Test Organisms

The mycobacteria strains, namely *Mycobacterium madagascariense* (MM) DSM 44641 and *Mycobacterium indicus pranii* (MIP) DSM 45239 supplied by the Germany Resource Centre for Biological Materials, Braunschweig, Germany. The two fast growing mycobacteria strains are isoniazid resistant and were used as markers for determination of a potential anti-TB efficacy of sterols.

2.6.2. Sub-culturing of Mycobacterium species

The strains were sub-cultured in Middlebrook 7H9 broth base supplemented with glycerol. The medium was prepared by suspending 1.18 g of Middlebrook 7H9 broth base in 230 ml of distilled water in a Scotch bottle (500 ml) followed by addition of 1 ml of glycerol (AR). The mixture was heated to dissolve the broth base completely, thereafter autoclaved at 121°C for 15 minutes. The mixture was left to cool to 31 and 35°C under lamina flow, before separately being inoculated with *Mycobacterium madagascariense* (*MM*) and *Mycobacterium indicus pranii* (*MIP*) respectively. Thereafter MM was incubated at 31 °C while MIP was incubated at 37 °C. The optimal growth of the bacteria cultures was observed after 5 days, and thus ready for antimycobacterial screening.

2.6.3. Determination of Minimum Inhibitory Concentration (MIC)

The MIC values of steroids against two *Mycobacterium* strains was determined by two fold microdilution method as described by Ellof, [7] and Erasto et al. [8].

2.6.4. Potentiation of antimycobacterial activity of isoniazid

Isoniazid (INH) has generally been found to be inactive against *Mycobacterium madagascariense* and *M. indicus pranii* even at higher concentration. This offers the opportunity to investigate the ability of sterols to potentiate the efficacy of INH against *MM* and *MIP*. Adopting the method of Ellof, [7] with modification, the fractional minimum inhibitory concentration (FMIC) of steroids were determined by screening 1/2 to 1/32 MIC values of alkaloids against *MM* and *MIP*, blended with 1/2 to 1/32 of the documented MIC value of isoniazid against *Mycobacterium tuberculosis*. This implied that, the first wells had 1/2 MIC values of a steroid and INH which was then diluted two folds to the last well which had 1/32 MIC values of test samples. The controls in this assay were as follow; two rows with steroid (1/2 to 1/32 MIC values), mycobacteria innoculums and broth only, two rows with INH, mycobacteria innoculum and broth only and positive control wells which had ciprofloxacin, mycobacteria innoculum and broth. The FMIC values of steroids was determined by addition of 40 µl (0.2 mg/ml) iodonitrotetrazolium (INT) chloride salt into each well and plates incubated at 31 (*MM*) and 37 °C (*MIP*) for 1 hour. The FMIC values were read at the concentration where a marked no change in color formation as a result of INT metabolism by active mycobacteria was observed.

2.6.5. Sub-culturing of M. madagascariense for bioassay guided isolation of steroids

The method described in section 2.6.2 was used to culture *M. madagascariense* used in the bioassay guided isolation of steroids. The full grown culture was maintained in a large glass Petri-dish and constantly incubated under 31°C. The developed TLC plate with clearly separated spots was dipped in the mycobacteria culture and thereafter incubated for 8 to 10 hours at 31°C and thereafter 0.2 mg/ml of iodonitrotetrazolium (INT) chloride salt sprayed on the plate and incubated for 1 hour. The clear zones of inhibition against purple background indicated bioactive compounds.

3. RESULTS AND DISCUSSION

3.1. Isolation of antimycobacterial steroids

The bioassay guided chromatographic isolation of compounds from aromatic stem sap of *Commiphora eminii* afforded two sterols namely; 4α-methylcholest-7-en-3β-ol (**1**) and Cholest-7-en-3β-ol (**2**) also commonly known as lathosterol (Figure 1). The chemical structures of the two secondary metabolites was established using NMR and MS spectroscopic analysis and with comparison with available data in literature [9]. An 4α-methyl sterol (**1**) has previously been identified as a minor sterol and important precursor in the biosynthesis of cholest-7-en-3β-ol (**2**) and other sterols in Echinoderms [9, 10]. Although sterol **1** has previously been reported as a minor sterol especially in marine organisms and in red fire ants *Solepsis invicta* [11], it is here reported for the first time in higher abundance from aromatic stem sap of *Commiphora eminii*.

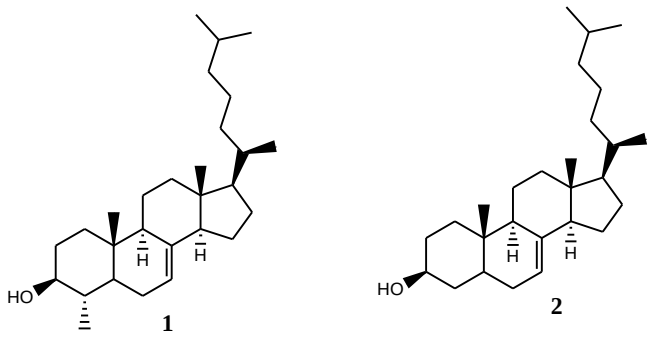

Fig. 1: Chemical structures of 4α-methyl-cholest-7-en-3β-ol (1) and cholest-7-en-3β-ol (2)

3.2. Antimycobacterial activity of sterols 1 and 2

The sterols were screened for antimycobacterial activity using two folds broth microdilution method. In this assay only cholest-7-en-3β-ol (**2**) exhibited moderate activity with MIC values of 1.6 mg/ml against both *M. madagascariense* and *M. indicus pranii* (Table 1). Sterol **1** lacked efficacy against all test organisms. The structural difference between two sterols is the presence of an alpha methyl group attached at carbon 4 of the 4α-methyl-cholest-7-en-3β-ol. This group may be responsible for the loss of activity in a sterol **1**. In this assay, Isoniazid (INH) a standard anti-TB drug lacked efficacy even at higher concentration. This observation corroborates the earlier reports that *M. madagascariense* and *M. indicus pranii* are resistant to isoniazid even at higher concentrations [12, 13]. Most sterols are lipophilic, thus are likely penetrate easily through a *Mycobacterium* cell wall, this biochemical property makes them suitable candidates for creating cellular pathway for drugs like INH to act on targets in the microbe cell. It is therefore possible that micro-molar concentration of sterols can enhance the activity of INH in the *Mycobacterium* cell.

3.3. Potentiation of antimycobacterial efficacy of isoniazid using sterols

The combination experiment of INH (1/2 to 1/32 MIC value of INH against Mtb) with micro-molar concentration of the sterols (ranging from ½ to 1/32 MIC value) was designed to investigate whether the advantage of sterols being able and fast penetrate the walls of organisms will potentiate antimycobacterial efficacy of INH. In this assay, there were three different combinations namely; i) INH and 4α-methyl-cholest-7-en-3β-ol (**1**), ii) INH and cholest-7-en-3β-ol (**2**) and iii) combination of sterols **1** and **2**. Since compound **1** lacked efficacy against test organisms, its concentrations in the combination experiments were based on the MIC values of **2**.

Table 1: Minimum Inhibitory Concentration (MIC) and Fraction Minimum Inhibitory Concentration (FMIC) of Sterols 1 and 2

Sterols and Combination of Sterol and Isoniazid	MIC (mg/ml)		FMIC (mg/ml)	
	MM	*MIP*	*MM*	*MIP*
4α-methyl-cholest-7-en-3β-ol (**1**)	NA*	NA		
1 + INH[a]			0.2	0.2
1 (1/2 to 1/32 of MIC value of **2**)[b]			NA	NA
Cholest-7-en-3β-ol (**2**)	1.6	1.6		
2 +INH			0.1	0.1
2 (1/2 to 1/32 of its MIC value)			NA	NA
Isoniazid (INH)	NA	NA		
Ciprofloxacin (μg/ml)	<5	<5		

[a]*Sterols combined with isoniazid at the range of ½ to 1/32 of their MIC values.*

[b]*Sterol **1** was inactive, therefore MIC value used was of Sterol **2***

**NA = No activity; MM = Mycobacterium madagascariense; MIP = Mycobacterium indicus pranii*

The sterols potentiated activity of isoniazid against resistant strains *M. madagascariense* and *M. indicus pranii*. A cholest-7-en-3β-ol **2** sterol potentiated activity of isoniazid at a fractional minimum inhibitory concentration (FMIC) of 0.1 mg/ml which is equivalent to 1/16 of its MIC value when tested alone (Table 1). When sterol **2** was screened alone in the same assay at the concentration range of 0.8 to 0.05 mg/ml (equivalent to ½ to 1/32 of the MIC value), it did not show any activity against test organisms. It was interesting to further observe that 4α-methyl-cholest-7-en-3β-ol (**1**) a sterol which lacked efficacy against the two mycobacteria strains in the first antimycobacterial assay; it potentiated activity of Isoniazid against test organisms. It exhibited a fractional minimum inhibitory concentration of 0.2 mg/ml against all test organisms. The combination of sterols **1** and **2** in the same concentration range, that is from 0.8 to 0.05 mg/ml lacked efficacy against test organisms (Table 1).

These results indicates that, although, sterols may be weak or moderately active against *Mycobacterium* strains, but can potentiate the activity of some potent anti-TB drugs such as isoniazid. This is because, the concentration of isoniazid used ranged from ½ to 1/32 of its MIC value against *M. tuberculosis*. This is quite low; therefore combination of Isoniazid with some sterols may be useful in the enhancement of its efficacy. Further work is however needed to find out the mode of action to which sterols potentiate activity of INH in mycobacteria, and possibly determine drug targets when combinations are used in the assay.

4. ACKNOWLEDGEMENTS

The author appreciates the financial support of The International Foundation for Science (IFS) Grant No. F/4894-1 and SIDA/MUHAS.

5. REFERENCES

1. Shen T, Wan W, Yuan H, Kong F, Guo H, Fan P, Lou H. *Phytochemistry*, 2007; **67**:1331-1337.
2. Birkett MA, Abasi SA, KrÖber T, Chamberlains K, Hooper AM, Guerin PM et al. *Phytochemistry*, 2008; **69**:1710-1715.
3. Dolara P, Corte B, Ghelardini C, Pugliese AM, Cerbai E, Menichetti S, Nostro AL. *Plant Med*, 2000; **66**: 356-358.
4. Habtemariam S. *Toxicon*, 2003; **41**: 723-727.
5. Francisca JA, Rajab SN, Nair MG. *Chem Biodivers*, 2004; **1**: 1842-1853.
6. Rulangaranga Z K. 1989. Some important indigenous medicinal and aromatic plants in the wild flora of Tanzania mainland. Tropical Forestry Action Plan, Working Paper 24. Tanzania Ministry of Lands, National Resources and Tourism, Dar-es-Salaam, Tanzania.
7. Ellof JN. *Planta Med*, 1998; **64**: 711-713.

8. Erasto P, Mbwambo ZH, Nondo RSO, Lall N. Lubschagne A, *Spatula DD*. 2011; **1**: 73-80.
9. Smith AG, Rubinstein I, Goad LJ. *Biochem J*, 1973; **135**: 443-455.
10. Smith AG, Goad LJ. *Biochem J*, 1975; **146**: 35-40.
11. Ba AS, Guo D-A, Norton RA, Phillips SA, Nes WD. *Arc Insect Biochem Phys*, 1995; **29**: 1-9.
12. Kadza J, Muller H-J, Stackebrandt E, Daffe M, Muller K, Pitulle C. *Intern J Sys Bacteriol,* 1992; **42**: 524-528.
13. Saini V, Raghuranshi S, Talwar GP, Ahmed N, Khurana JP, Hasnain SE et al. *PlosONE*, 2009; **4**: 1-10.