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Research Article

Antimicrobial Activity of β-Sitosterol Isolated from *Kalanchoe tomentosa* Leaves Against *Staphylococcus aureus* and *Klebsiella pneumonia*

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Abstract

Background and Objective: *Kalanchoe tomentosa* is identified and their different characteristics regarding the antibacterial and antioxidant properties have a vast effect. Fresh *K. tomentosa* leaves obtained from Bandung, Indonesia was extracted using n-hexane followed by serial dichloromethane maceration. **Materials and Methods:** N-hexane and ethyl acetate were used to separate the dichloromethane extract using vacuum liquid chromatography and the isolated compounds were recrystallized with n-hexane. **Results:** About 37 mg of dichloromethane extract was obtained from the extraction process. Recrystallized compound isolates were identified as stigmast-5-en-3-ol or β-sitosterol. Both dichloromethane extract and β-sitosterol isolated compounds showed strong bacteriostatic activity against *S. aureus* with MIC = 15.63 and 7.81 μg mL^{-1} and *K. pneumonia* with MIC = 7.81 and 31.25 μg mL^{-1}, respectively. However, only dichloromethane extract exhibited a bactericidal effect (7.81 μg mL^{-1}). **Conclusion:** The pure β-sitosterol compound was isolated from *K. tomentosa* dichloromethane extract. Both the dichloromethane extract and the isolated β-sitosterol compound had antibacterial effects against *S. aureus* and *K. pneumonia*.

Key words: *Kalanchoe tomentosa*, β-sitosterol, oral infection, minimum inhibition concentration, minimum bactericidal concentration, cephalosporins, bacterial pathogen, lymphadenitis

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Oral cavity infections are often caused by both aerobic and anaerobic bacteria. Odontogenic infections such as osteomyelitis can cause symptoms such as oedema, pain, lymphadenitis, fever, cellulitis and trismus[1,2].

Staphylococcus aureus is a bacterial pathogen responsible for a wide range of infections[3]. It can survive prolonged extreme conditions to cause infection, it causes abscesses when neutrophils enter the infection site in the invasion stage and can directly invade the lymph vessels and blood to produce bacterial toxins that cause severe infection[4,5]. Invasion of *S. aureus* into the heart tissue can cause acute endocarditis. *Staphylococcus aureus* strains are resistant to several antibiotics including methicillin, nafcillin and cephalosporins. These strains are known as methicillin-resistant *S. aureus* (MRSA)[6]. The presence of *S. aureus* is confirmed via blood culture where whitish-gold colonies are indicative of the pathogen. A clear halo around the bacterial colony is characteristic of this bacterium's release of haemolysin A toxin which ruptures red blood cells[7].

Klebsiella pneumoniae is a gram-negative bacterium. Barlean *et al.* reported *K. pneumoniae* and *S. aureus* as the most commonly identified pathogens in surgical site infections in oral and maxillofacial surgery patients[8]. As with *S. aureus*, *K. pneumonia* also exhibits antibiotic resistance[9,10]. Both bacteria are present in the oral cavity and have been reported in postoperative head and neck wound infections.

Adequate antibacterial administration is needed to treat bacterial infections. However, increased resistance with widespread antibacterial use has become a major public health issue worldwide. Therefore, it is necessary to search for novel antibacterial agents to treat infections without causing resistance. Natural ingredients have been a promising target for new antibacterial agents with fewer side effects[4,8].

Kalanchoe is a large genus of colourful succulent plants. It grows widely in Africa, Saudi Arabia, Asia, the Americas and Australia[11,12]. Some species of Kalanchoe are used as traditional medicinal plants. *Kalanchoe tomentosa* is part of the family Crassulaceae, it is a succulent plant with dense, white, hair-like covering. It is also commonly known as the Panda plant. *K. tomentosa* is rich in alkaloids, triterpenes, glycosides, flavonoids, steroids and lipids[12-14]. Ethanolic extracts of *K. tomentosa* (Crassulaceae) has been reported to contain 14 compounds including α-amyrin acetate, friedelin, glutinol, 1-dotriacontanol, phytol, stigmasta-7,25-dien-3b-ol, β-sitosterol, isorhamnetin, 2,3-dihydroxypropyl tetradecanoate, eriodictyol, gallic acid, quercetin, kaempferol-3-O-Rutinoside and isovitexin[12]. The flavonoid profile of *K. tomentosa* increases cytotoxic activity against P-388 murine leukaemia cells[13]. However, literature reporting the antibacterial activity of *K. tomentosa* remains limited.

β-sitosterol is a bioactive phytosterol that is naturally derived from plant cell membranes[15]. It has been reported to exhibit antibacterial activity against *S. aureus* and *Escherichia coli*. Furthermore, it has been reported to show interesting anti-inflammatory and wound healing effects[15]. Pneumolysin is a toxin released by *S. pneumolysin* that is not targeted by currently available antibiotics, making it an interesting target for the development of therapeutics against this pathogen[16]. The phytosterol β-sitosterol has been shown to effectively protect against pneumolysin-induced cell lysis. β-sitosterol interacts with the toxin at Thr459 and Leu460.

This *in vitro* study aims to determine the potential of *K. tomentosa* extracts against *S. aureus* and *K. pneumoniae* bacteria, compared to β-sitosterol standards.

MATERIALS AND METHODS

Study area: The study was carried out in the Laboratory of Chemistry, Department of Chemistry, Faculty of Sciences and Informatics, Jenderal Ahmad Yani University, Cimahi, Indonesia from 2019-2020.

Chemical and equipment: A set of maceration and KCV. Aqueous sterile, acetone (CH_3COCH_3), ethyl acetate ($CH_3COOC_2H_5$), dichloromethane (CH_2Cl_2), methanol (CH_3OH), n-hexane (C_6H_{14}), chloroform ($CHCl_3$) p.a, Thin Layer Chromatography (TLC) plate F254 Merck Germany, Silica gel Merck 60 (0.2-0.5 mm) Germany, Silica gel Merck 60 G Ultraviolet lamps, brand Vilber Lourmat VL-8. LC, rotary evaporator brand Heidolph Laborota 4000, a set of vacuum liquid chromatography (VLC), Ultraviolet spectrophotometer; Hewlett Packard 8453, Infrared spectrophotometer, Shimadzu Type IR Prestige-21, spectrometer; NMR ^1H brand JEOL Type JNM-ECA 500 MHz methods were used to elucidate the structures of β-sitosterol.

Plant collection, extraction and isolation of compounds: *Kalanchoe tomentosa* leaves were obtained from a nursery at Lembang, Bandung, West Java, Indonesia (6.8145°S latitude, 107.6230°E longitude, 2.254 ft elevation). The plant was transported to our laboratory in Bandung city and was identified by an expert before being cleaned and cut into smaller pieces.

About 20 kg of fresh *K. tomentosa* leaves were ground and extracted via maceration using n-hexane for 24 hrs. Maceration was repeated until the extract was colourless (indicated using thin-layer chromatography (TLC)). The extract was filtered and re-extracted using methylene chloride for 24 hrs. The final extract was filtered and concentrated using a rotary evaporator. A solid dark green methylene chloride extract (MCE) was separated using vacuum liquid chromatography (VLC) (silica gel G60) with n-hexane-EtOAc solvent. The TLC using n-hexane-EtOAc was performed on the crystals chosen to determine their purity and compare them against pure β-sitosterol compounds.

Antibacterial activity test: About 100 µL nutrient broth (NB) was added into the first column of a 96-well plate as the negative control. About 5 µL of *S. aureus* and *K. pneumonia* bacterial suspensions was added into 10 mL NB and vortexed to mix. About 100 µL of the bacterial suspension was added to columns 2-12. About 100 µL of MCE5 was added to each well and pipetted to mix. Next, 100 µL was taken from the second column and serially diluted across the 3rd column until the 12th column. The plate was incubated at 37°C for 24 hrs and then the clear wells were observed. The lowest concentration where no microbial growth was detected was defined as the minimum bactericidal concentration (MBC). About 5 µL of solution from the clear wells were transferred into nutrient agar (NA) and incubated at 37°C for 24 hrs. The lowest concentration where no microbial growth was observed was defined as minimum inhibitory concentration (MIC).

RESULTS

Extraction and Isolation of compounds: The final extract was filtered and concentrated using a rotary evaporator to yield 35 mg of a solid dark green methylene chloride extract (MCE), then separation using VLC with n-hexane-EtOAc solvent obtained 10 fractions. The fifth fraction (MCE5) produced 8 mg of white needle crystals when re-crystallized with n-hexane. The MCE5 was determined the purity and compared against pure β-sitosterol compounds.

UV and TLC analysis: The UV spectroscopy analysis of MCE5 was detected at 264 and 364 nm. The TLC analysis of the MCE5 against the β-sitosterol standard is shown in Fig. 1.

^{1}HNMR and IR analysis: Analysis of the first isolate using ^{1}HNMR (500 MHz, CDCl3) is shown in Table 1. Only one signal was visible above δH 5 ppm and visible signals accumulated in areas below δH 2 ppm.

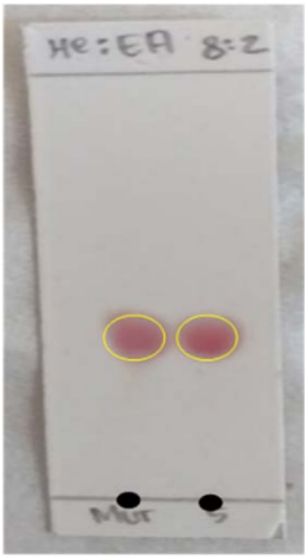

Fig. 1: TLC analysis of MCE5 (left) against β-sitosterol standard (right) Both showed similar Rf values

Table 1: ^{1}HNMR data comparison between *K. tomentosa* MCE5 and β-sitosterol

Position of atom C	^{1}HNMR δH (ppm) (ΣH:mult: J = Hz)	
	K. tomentosa MCE5	β-sitosterol*
1	0.82 : 0, 86 (2H, dd, 10,5 : 5.5)	1.07 : 1.02 (2H, dd, 10.5 :5.5)
2	1.44 : 1.47 (2H, dd, 9.5 : 6,0)	1.44 : 1.48 (2H,td,9.5: 6.0)
3	3.49 : 3.55 (1H, m)	3.51 (1H,m)
4	2.23 : 2.31 (2H, m)	2.22 : 2.29 (2H,m)
5	-	-
6	5.35 (1H,t, 2.5)	5.35 (1H,br)
7	2.01 (2H, dt, 5,6 : 8,5)	1.85 : 2.01 (2H,dt, 5.6: 8.5)
8	1.60 (1H, m)	1.57 (1H,m)
9	0.93 (1H, m)	0.93 (1H,m)
10	-	-
11	1.42 : 1.47 (2H, m)	1.42 : 1.49 (2H,m)
12	1.95 (2H, d, 5.6)	1.15 : 1.98 (2H,d, 5.6)
13	-	-
14	1.01 (1H, m)	1.00 (1H,m)
15	1.94 : 2.03 (2H, m)	1.57 (2H,m)
16	1.95 (2H, m)	1.84 (2H, m)
17	1.09 (1H, dt, 5.2 : 8.5)	1.09 (1H,dt, 5.2: 8.5)
18	0.68 (3H, s)	0.68 (3H,s)
19	1.01 (3H, s)	1.01 (3H,s)
20	1.31 (1H, m)	1.36 (1H,m)
21	0.69 (3H, d : 6,1)	0.92 (3H,d, 6.1)
22	1.80 (2H, m)	1.38 (2H,m)
23	1.50 (2H, m)	1.54 (2H,m)
24	: 0,98 (1H, m)	0.93 (1H,m)
25	1.66 (1H, m)	1.66 (1H,m)
26	0.84 (3H,d, 6.2)	0.84 (3H,d, 6.2)
27	(3H,d, 6,7)	0.92 (3H,d, 6.7)
28	1.25 (2H, m)	1.26 (2H, m)
29	0.82 (3H,s)	0.83 (3H,s)

Table 2: IR data comparison between *K. tomentosa* MCE5 and β-sitosterol

Isolate 1 (cm^{-1})	β-sitosterol* (cm^{-1})	Creswell** (cm^{-1})	Functional group
3415.93	3440.62	3450-3200	O-H
2945.30	2936.69	2800-3000	C-H aliphatic
1649.14	1646.55	1680-1620	C=C
1454.33	1463.42	1475-1300	C-H (in CH_2)
1371.39	1381.65	1475-1300	C-H (in CH_3)
1047.35	1053.89	1050-1260	C-O alcohol
962.48	970.32	995-710	=CH alkene

**Creswell dkk., 1982 and *Fitriani, 2013

Table 3: Antimicrobial activity of MCE5 and β-sitosterol against *S. aureus* and *K. tomentosa*

	MCE5		β -sitosterol	
	MIC (μg mL^{-1})	MBC (μg mL^{-1})	MIC (μg mL^{-1})	MBC (μg mL^{-1})
S. aureus	15.63	-	7.81	-
K. pneumonia	7.81	7.81	31.25	-

The various functional groups in MCE5 were elucidated using infrared (IR) spectrophotometry. The results of IR spectral data of MCE5 show absorption in the following areas: 3415 cm^{-1} area with widening intensity. Absorption at 1454 and 1371 cm^{-1} with a sharp intensity indicated a C-H function group in CH2 and CH3 as shown in Table 2.

Antimicrobial activity: The MIC values of MCE5 against *S. aureus* and *K. pneumonia* with were 15.63 and 7.81 μg mL^{-1}, respectively. The MBC values are 7.81 μg mL^{-1} as shown in Table 3.

DISCUSSION

The UV spectroscopy analysis of MCE5 indicated strong absorption at 264 nm and weak absorption at 364 nm. The values indicate the presence of an unconjugated alkene system and the absence of aromatic systems. The TLC analysis of the MCE5 against the β-sitosterol standard showed similar Rf values (Fig. 1). Based on the values, it was predicted that MCE5 to be β-sitosterol. The above result is reinforced by data from ^{1}HNMR analysis. Analysis of the first isolate using ^{1}HNMR (500 MHz, $CDCl_3$) proved that the isolate was a steroid compound, as shown in Table 1. There was a distinctive oleophilic proton signal at δH 5 ppm and an oxygenated proton signal at δH 3 ppm, which is commonly found in the steroid groups[17].

In addition, a chemical shift in δH 1.42 ppm was characteristic of a cyclohexane group in ring A, B and C in steroid compound[18]. Proton signals found at δH 0.86 (2H, dd), 1.44 (2H, dt), 2.23 (2H, m), 2.01 (2H, dt), 1.42 (2H, m), 1.99 (2H, d), 1.94 (2H, m), 1.95 (2H, m) are signals for methylene protons (CH_2). Proton signals at δH 3.49 (1H, m), 5.35 (1H, t), 1.60 (1H, m), 0.93 (1H, m), 1.01 (1H, m), 1.09 ppm (1H, dt) indicate the methine proton, δH 3.49 (1H, m) indicates the presence of hydrogen adjacent to the hydroxyl group suspected in C-3 in ring A present next to the molecular plane, on the same side as the methyl groups in C-10 and C-13. This configuration is recognized as -configuration. A proton signal at δH 5.35 (1H, t) shows oleophilic methine, indicating a double bond in C-5[17]. Each proton signal at δH 0.68 (3H, s) and 1.01 (3H, s) correspond to methyl groups as steroid substituents in the main framework at C-10 and C-13, respectively. These three signals (methylene, methine and methyl) indicate the presence of a steroid framework[19] substituted by two methyl and one hydroxyl group.

Signals were observed in the aliphatic region (substituent at C-17) that indicate an alkane unit: Three signals for the methylene group at δH 1.80 (2H, m), 1.50 (2H, m) and 1.25 (2H, m), three signals for the methine group at δH 1.31 (1H, m), 0.94 (1H, m), 1.66 (1H, m) and four methyl group signals at δH 0.69 (3H, d), 0.84 (3H, d), 0.92 (3H, d) and 0.82 (3H, d). These ten proton signals correspond to alkyl skeletons[20]. All the shifts were compared against β-sitosterol 10 compounds and many similarities were found. It was therefore concluded that MCE5 was a β-sitosterol compound.

The various functional groups in MCE5 were elucidated using infrared (IR) spectrophotometry. The results of IR spectral data of MCE5 show absorption in the following areas: 3415 cm^{-1} area with widening intensity, indicating alcohol group (-OH). This is reinforced by absorption at 1047 cm^{-1} with sharp intensity indicating alcohol (C-O), absorption at 2945 cm^{-1} with sharp intensity indicating aliphatic functional group (CH), absorption at 1649 cm^{-1} with widening intensity indicative of carbon group (C = C), absorption at 835 cm^{-1} with moderate intensity indicating alkenes (=CH)[21]. According to Shaleh *et al.*[12], absorption at 1649 cm^{-1} indicates the existence of a range of C=C functional groups with no conjugation that has a long-range wave of between 1620-1680 cm^{-1}.

The IR spectral data results reinforce that the isolates obtained are a group of steroid compounds that do not have conjugated double bonds. Absorption at 1454 and 1371 cm^{-1} with a sharp intensity indicated a C-H function group in CH_2 and CH_3. The MCE5 IR spectral data results were compared against pure β-sitosterol compounds. Similar absorption patterns were identified, as shown in Table 2.

Results of TLC, 1HNMR and IR analysis of MCE5 indicate that MCE5 from *K. tomentosa* may be a β-sitosterol compound, specifically, compound IUPAC stigmast-5-en-3β-ol that belongs to the group stigmata in originating hydrocarbons steroid with the molecular formula $C_{29}H_{50}O$.

The MCE5 had bacteriostatic effects against *S. aureus* and *K. pneumonia* with MIC values of 15.63 and 7.81 μg mL^{-1}, respectively. The MCE did not have a bactericidal effect against *S. aureus*, but did against *K. pneumonia* with MBC of 7.81 μg mL^{-1}. Therefore, MCE5 had strong activity against *S. aureus* and *K. pneumonia*, as shown in Table 3.

The MIC data show *K. pneumonia* required lower doses of MCE5 and β-sitosterol compared to *S. aureus* for effective antibacterial activity. Gram-positive and Gram-negative bacteria have differences in their cell wall structures, which affect their susceptibility to antibacterial agents. Gram-positive bacterial cell walls have a single-layered peptidoglycan structure that is polar and has low lipid content (Pelczar). They also contain polysaccharides, which serve as positive ion transfers in and out of the cell. Hence, gram-positive bacterial cell walls are more polar and more susceptible to antibacterial agents.

The MCE5 had one hydroxyl group (-OH) in its structure. The polar -OH group can penetrate the polar peptide and damage the bacterial cell wall by severing the peptidoglycan bonds to compromise the cell layer. This leaves the cytoplasmic membrane vulnerable to damage, causing the leak of important metabolites and activation of the bacterial enzyme system. Antibacterials target the peptidoglycan layer of cell walls in the bacteria. This layer is essential in preserving the bacteria from hypotonic environments, hence, damage or loss of this layer will lead to loss of cell wall strength, resulting in death[22]. Further research is needed to identify the other secondary metabolite compounds present in K. tomentosa, as well as elucidation of the structure of the compound via analysis using MS, CNMR and two- and three-dimensional NMR. Isolation and identification of pure β-sitosterol compounds in the fifth fraction of the methyl chloride extract from the leaves of *K. tomentosa* conferred bacteriostatic effects against *S. aureus* and *K. pneumonia*.

CONCLUSION

The pure β-sitosterol compound was isolated from *K. tomentosa* dichloromethane extract. Both the dichloromethane extract and the isolated β-sitosterol compound had antibacterial effects against *S. aureus* and *K. pneumonia*.

SIGNIFICANCE STATEMENT

This study discovers the β-sitosterol compound was isolated from *K. tomentosa* dichloromethane extract as one option of antimicrobial drug discovery. This study will help the researcher to hone the *Kalanchoe* genus elucidation using a different method of extraction that there was still very few papers on it. Thus, possibly other advantageous combinations of the Kalanchoe may be arrived at.

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