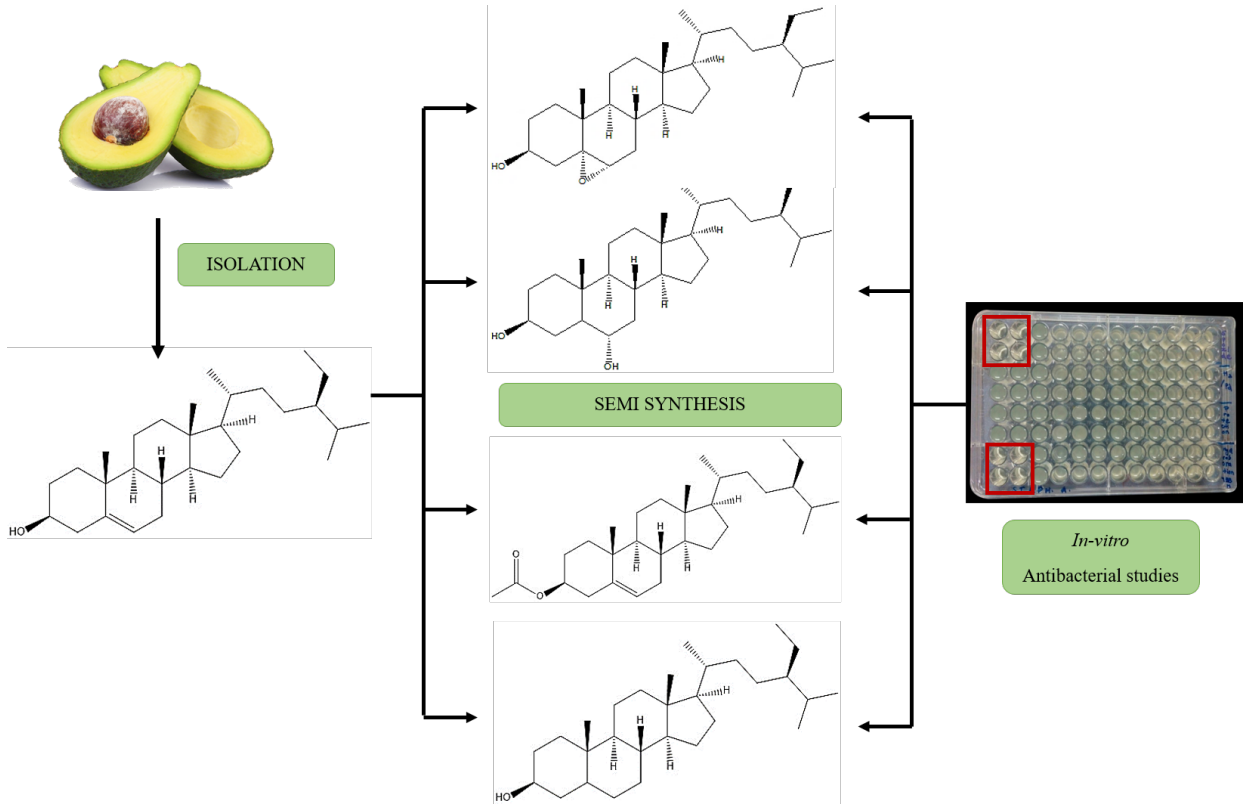
RESEARCH ARTICLE

# Synthesis of derivatives of *β*-sitosterol and evaluation of their anti-bacterial activity

**L.A.M. Fernando, W.G.D. Wickramasingha and S. Jayasinghe***




## Highlights

- *β*-Sitosterol was isolated from ripen fruits of Avocado with a relatively good yield.
- Four derivatives were synthesized and C6 hydroxy *β*-sitosterol was reported for the first time.
- The anti-bacterial activity was reduced for derivatives with increasing lipophilicity.
- Hydroxyl groups at C3 and C6 are significant towards the antibacterial activity.

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# Synthesis of derivatives of *β*-sitosterol and evaluation of their anti-bacterial activity

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**Abstract**: Antimicrobial resistance is one of a major health issue and warrants the development of new antimicrobial agents with structural diversity and novel mechanisms of action. *β*-Sitosterol is naturally abundant steroid in many plant families that shows moderate antibacterial activity. Hence, this study aimed to identify the importance of the functional groups present in *β*-sitosterol in enhancing the antibacterial activity. *β*-Sitosterol was isolated from the dichloromethane extract of fruit pulps of *Persea americana* (Yield 2.3% with respect to the crude). Isolated *β*-sitosterol was subjected to epoxidation, hydrogenation, and anti-Markovnikov hydration at the endocyclic olefin at C5-C6, and the acetylation at C3- hydroxyl group. The parent compound and semisynthetic derivatives were characterized using $^{1}$H-NMR and FTIR and their antibacterial activity was determined using the micro broth dilution method with respect to the oxacillin and amoxicillin. The derivative with C6 hydroxyl of *β*-sitosterol was reported for the first time. The minimum inhibition concentrations of *β*-sitosterol, epoxide, and C6 hydroxyl compound were 512 ppm, and that of hydrogenated and acetylated compounds were above 1024 ppm. These empirical data revealed that the incorporation of oxygen heteroatom at the C5 and C6 positions while removing the double bond character at C5-C6 has not affected the antibacterial activity of *β*-sitosterol, however, hydrogenation and acetylation at the C3 hydroxyl group have significantly reduced the antibacterial activity compared to the *β*-sitosterol.

***Keywords***: *β*-sitosterol; epoxidation; anti-Markovnikov hydration; hydrogenation; acetylation; antibacterial activity.

## INTRODUCTION

Bacterial infections are one of the major global health problems due to the growing resistance of bacteria to the available antibiotics as a result of misuse and overuse of them. Especially it is a heavy burden for developing and low-income countries as many patients with resistant bacterial infections are difficult to be cured and hence increasing the morbidity and mortality while causing a further escalation of the cost of health care delivery (Bygbjerg, 2011; Coker *et al*., 2011). Hence exploring the avenues for new antibiotics is always at utmost importance to overcome the issues related to resistant bacterial infections.

Natural products and their derivatives play a huge role in exploring new antibiotics owing to their vast structural diversity, metabolic stability, and ability of being used as safe and broadly effective alternatives with less adverse effects (Sasidharan *et al*., 2011). Many new antibiotics have been developed through the chemical modification of natural compounds such as *β*-lactams, carbapenems, and cephalosporin. Approximately, one-third of the top-selling drugs in the world are natural products or their derivatives (Newmann and Gordon, 2016). But with the identification of antibiotic-resistant bacterial strains towards the available standard antibiotics scientists put their focus on synthesizing derivatives of natural products which are already being used as antibiotics. A better example for this is the very first glycopeptide antibiotic; vancomycin which was the most commonly used drug in the treatment of Gram-positive bacterial infections that were resistant to common antibiotics, some decades ago. As vancomycin-resistant bacterial strains emerged, derivatives of vancomycin had been synthesized and tested for the antibacterial activity for vancomycin-resistant strains. Nagarajan *et al*. (1993) and Ge *et al*. (1999) have reported that carbohydrate derivatives of vancomycin are more active against vancomycin-resistant bacterial strains and hence serve as a great solution for antibiotics resistance. Consequently with these findings and subsequent efforts of the number of other scientists, a semi-synthetic derivative of vancomycin; telavancin was approved in 2007 for methicillin-resistant bacterial strains (Food and Drug Administration, United States). However, the development and introduction of new antibiotics and observation of resistant organisms would be a never-ending process.

Synthesis of derivatives of common natural anti-bacterial compounds would not only explain the structure-activity relationships of their inherent properties but also suggests the potential alternatives that could be effective in the treatment of these resistant bacterial infections. The present study was carried out as a preliminary step towards finding potent antibiotic drug leads using *β*-sitosterol which is abundant in many plant families.

*β*-Sitosterol (compound 1) is a steroid that has a chemical structure similar to cholesterol (Figure 1) and has been easily isolated from fruits such as avocado, vegetable oils, and nuts such as soybeans. It is reported to have diverse bio-activities such as anti-inflammatory activity,

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hypocholesterolaemic activity, anti-cancer activity, and angiogenic activity (Saeidnia *et al*., 2014). Although many scientists claim that *β*-sitosterol has potential antibacterial activity (Sanches *et al*., 2005; Sen *et al*., 2012), some of them has proven that antibacterial activity of *β*-sitosterol is less when compared with standard antibiotics (Chandramu *et al*., 2003; Li *et al*., 2008; Kongkathip *et al*., 2009). Even though there are controversial statements on its antibacterial activity, its natural abundance and inherited various biological activities made our interest to produce semisynthetic derivatives of *β*-sitosterol in order to enhance the antibacterial activity and to study the structure-activity relationship. Four different derivatives of *β*-sitosterol were synthesized by either modifying the double bond at the C5-C6 by epoxidation (2), hydrogenation (3), and anti-Markovnikov hydration (5), or the hydroxyl group at C3 by acetylation (4) in our study. Antibacterial activities of *β*-sitosterol and its semisynthetic derivatives (compounds 1-5) were evaluated using the micro broth dilution method as per the procedure given by the Clinical and Laboratory Standards Institute (CLSI, 2018).

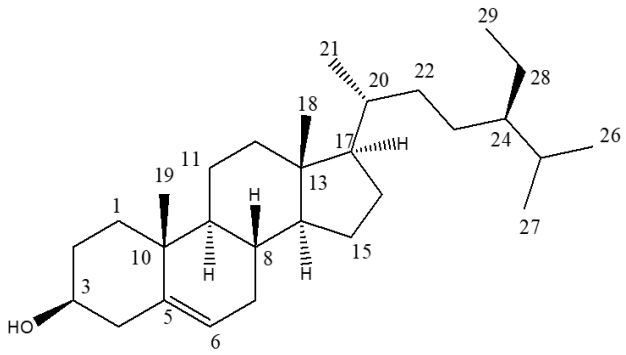



**Figure 1:** Chemical structure of Compound 1.

## MATERIALS AND METHODS

General $^1$H-NMR spectra were performed on a Varian Mercury 300 MHz using $CDCl_3$ as the solvent and infrared spectra were recorded on an FTIR Perkin Elmer spectrophotometer. Flash Column Chromatography (FCC) refers to chromatography carried out through columns packed with Euclid silica gel 60 (0.040 - 0.063 mm). Fractions from FCC subjected to Thin Layer Chromatography (TLC) with 0.1 mm thick 60F 254 Kiesel G. MERCK plates with art 230 - 400 ASTM silica gel as stationary phase and with dichloromethane (DCM)/hexane (6/4) as eluent and visualized with anisaldehyde-sulphuric acid followed by heating.

### Preparation of plant extract

*Persea americana* fruits were purchased from the market in Kandy, Sri Lanka. Fruit pulp was freeze-dried in order to remove water and a 50 g sample was macerated for 3×24 h in DCM (300 mL) with constant shaking. A well-refined solution was filtered and concentrated under reduced pressure at 30 °C.

### Isolation of (3S,8S,9S,10R,13R,14S,17R)-17-[(2R,5R)-5-ethyl-6-methylheptan-2-yl]-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-ol (Compound 1)

The DCM extract (6.187 g) was fractionated using FCC with gradient solvent systems of hexane and DCM. The fractions were subjected to TLC and those that contained the targeted compound whose $R_f$ value (0.58, 60% DCM: hexane) was matched with the authentic sample were combined together and again subjected to FCC under isocratic elution with 60% DCM: hexane. The test tubes which contain pure compound were combined and characterized for structure elucidation.

The isolated compound was obtained as white needle like crystals. Comprehensive analysis of FTIR and $^1$H-NMR (Praveen *et al.*, 2011) of the isolated compound confirmed that it is *β*-sitosterol. FTIR (ν max cm$^{-1}$): 3437 (-OH), 2877 and 2954 (aliphatic -CH), 1654 (-C=C), 1052 (-C-O). $^1$H-NMR (δ ppm): 0.62-0.84 (m, 13H), 0.88 (s, 3H), 0.93-1.00 (d, *J* = 6.8 Hz, 3H), 1.03-1.31 (m, 5H), 1.34-2.09 (m, 19H), 2.15 (m, 1H), 2.22 (dd, *J* = 14.5, 2.3 Hz, 1H), 2.26 (dd, *J* = 14.5, 2.3 Hz, 1H), 3.51 (H3, m, 1H), 5.33 (H6, m, 1H).

### Synthesis of ([3S,4aS,5aR,6aS,6bS,9R,9aR,11aS,11bR]-9-[(2R,5R)-5-ethyl-6-methylheptan-2-yl]-9a,11b-dimethylhexadecahydrocyclopenta[1,2]phenanthro[8a,9-b]oxiren-3-ol) (Compound 2)

Compound 2 was synthesized according to the modified procedure described by Rega *et al*. (2007). To a solution of compound 1 (30 mg, 0.0723 mmol) in DCM (0.511 mL) at 0 °C was added *m*-chloroperbenzoic acid (*m*-CPBA) (32.439 mg, 0.1879 mmol) in DCM (0.511 mL) dropwise under $N_2$ atmosphere. The mixture was allowed to warm to room temperature and stirred for 20 h, then it was cooled to 0 °C and $Na_2SO_3$ (10%, 2.55 mL) was added, and the mixture was kept at this temperature for 6 h. Then the aqueous phase was extracted with DCM (2 mL×3). The combined organic extracts were washed with water (4 mL), dried with $Na_2SO_4$, and concentrated under reduced pressure. The residue was flash chromatographed using 70% DCM in hexane to give compound 2 as a single isomer (27.9 mg, 93%).

Compound 2 was obtained as white needle like crystals. FTIR (ν max cm$^{-1}$): 3444 (OH), 2814, and 2931 (aliphatic -CH), 1018 (-C-O). $^1$H-NMR (δ ppm): 1.29-0.64 (m, 25 H), 1.95 (m, 7H), 2.08 (H7, m, 2H), 2.91 (H6, dd, *J* = 6.6, 1.3 Hz, 1H), 3.93 (H3, m, 1H).

### Synthesis of [(3S,8R,9S,10S,13R,14S,17R)-17-[(2R,5R)-5-ethyl-6-methylheptan-2-yl]-10,13-dimethylhexadecahydro-1H-cyclopenta[a]phenanthren-3-ol] (Compound 3)

Compound 3 was synthesized according to the modified procedure described by Augustine and Reardon, (1969). A mixture of compound 1 (30 mg, 0.0723 mmol) in anhydrous methanol (2.79 mL) was hydrogenated over palladium on

charcoal (1.5 mg, 5%) at room temperature. After 10 h, the catalyst was removed by filtration and the solvent was evaporated under reduced pressure and the residue was passed through a column using 60% DCM: hexane to give compound 3 (29.1 mg, 96%).

Compound 3 was a white crystalline compound. FTIR (ν max $cm^{-1}$): 3464 (-OH), 2765 and 2985 (aliphatic -CH), 1014 (-C-O). $^1$H-NMR (δ ppm): 0.87 - 1.20 (m, 18 H) 1.27 (s, 6 H) 1.39 (s, 13 H) 1.77 (s, 10 H) 2.07 (s, 1 H) 3.77 (H3, m, 1 H).

**Synthesis of [(3S,8S,9S,10R,13R,14S,17R)-17-[(2R, 5R)-5-ethyl-6-methylheptan-2-yl]-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl acetate] (Compound 4)**

Compound 4 was synthesized according to the modified procedure described by Rali *et al*. (2016). To a mixture of compound 1 (30 mg, 0.0723 mmol.) in pyridine (1 mL) was added acetic anhydride (1.5 mL) in a 50 mL round bottom flask. The mixture was stirred for 12 h at room temperature. The product was poured into 5 mL of water and stirred for 2 h to hydrolyze excess acetic anhydride. Then the aqueous phase was extracted with DCM (2 mL×3). The combined organic extracts were washed with water (4 mL), dried with anhydrous $Na_2SO_4$, filtered, and concentrated under reduced pressure. The residue was subjected to FCC using 60% DCM: hexane to give compound 4 (29 mg, 96%).

Compound 4 was a white needle like crystalline compound. FTIR (ν max $cm^{-1}$): 2927 (aliphatic -CH), 1751 (-C=O), 1654 (-C=C), 1276 (-C-O). $^1$H-NMR (δ ppm): 0.86 (s, 9 H), 1.28 (m, 31 H), 1.86 (s, 4 H), 2.05 (s, 5 H), 2.33 (H7, m, 2 H), 4.62 (H3, m, 1H), 5.39 (H6, s, 1H).

**Synthesis of [(3S,8S,9S,10R,13R,14S,17R)-17-[(2R,5R)-5-ethyl-6-methylheptan-2-yl]-10,13-dimethylhexadecahydro-1H-cyclopenta[a]phenanthrene-3,6-diol] (Compound 5).**

Compound 5 was synthesized according to the modified procedure described by Bracher and Litz, 1996. To a mixture of compound 1 (30 mg, 0.0723 mmol) in THF (1 mL) at 0 °C was added 9-borabicyclo (3.3.1) nonane (9-BBN) (434 µL, 0.2169 mmol) dropwise with stirring over a period of 5 min. The reaction mixture was stirred at room temperature for 45 min. Then NaOH and $H_2O_2$ (7.45 µL) were added, stirred for a few minutes, and extracted with ethyl acetate (2 mL×3). The combined organic extracts were washed with 1% HCl to remove any NaOH and then with water to remove any trace of acid, dried with $Na_2SO_4$, filtered, and concentrated under reduced pressure. The residue was purified by FCC using solvent combinations of DCM, hexane, and ethyl acetate to give compound 5 (21 mg, 70%).

Compound 5 was a light yellow needle like crystalline compound. FTIR (ν max $cm^{-1}$): 3444 (-OH), 2970 (aliphatic -CH), 1060 (-C-O). $^1$H-NMR (δ ppm): 1.03 (s, 4 H), 1.07-1.23 (m, 6 H), 1.28 (s, 4 H), 1.32-1.52 (m, 12 H), 1.62-1.78 (m, 16 H), 1.90 (m, 2 H), 2.08-2.41 (m, 1 H), 3.82-3.85 (H3, H6, m, 2 H).

**Evaluation of antibacterial activity**

*β*-Sitosterol (compound 1) and its semisynthetic derivatives (compounds 2-5) were tested *in-vitro* for their antibacterial activity against standard strains of *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 25922 to represent Gram-positive and Gram-negative organisms, respectively, using micro broth dilution method according to the guidelines given by Clinical and Laboratory Standards Institute (CLSI, 2018)

**Preparation of bacterial cultures**

The turbidity of overnight cultures of each organism was adjusted to 0.5 McFarland standards and diluted 1:20 with sterile normal saline before inoculation ($5 \times 10^6$ CFU $mL^{-1}$) and it was administrated to the test plate within 15 minutes.

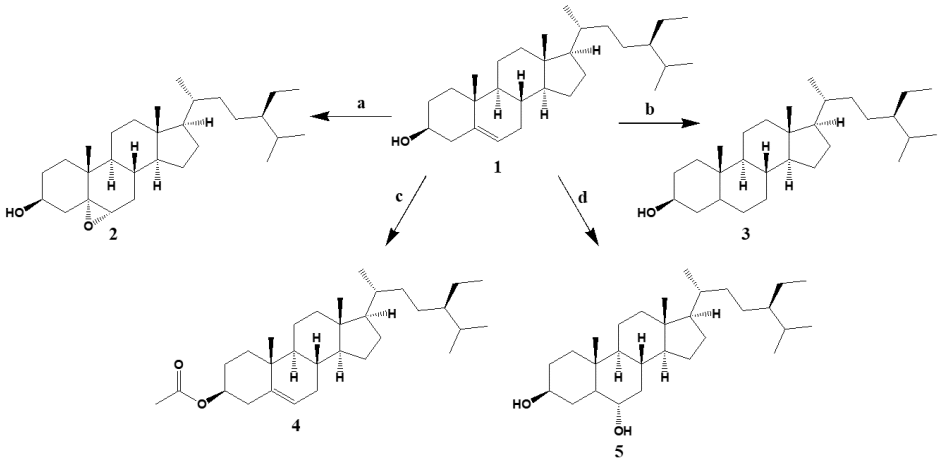
**Antibacterial susceptibility test**

*β*-Sitosterol (5.488 ± 0.001 mg ) was dissolved in 1000 µL of ethanol and diluted with 4 mL of Mueller-Hinton Broth (MHB) to obtain a 1024 ppm solution. Then a serial two-fold dilution with MHB was made up to 0.5 ppm in sterilized test tubes. The same procedure was carried out for the four semi synthesized derivatives. 96-Well plates were labelled and each test sample (190 µL) was added to appropriate wells followed by 10 µL of the bacterial suspension. The plate was then incubated at 37 °C for 18 - 24 h and turbidity formation was assessed visually. The lowest concentration at which no visual growth observed was taken as the Minimum Inhibition Concentration (MIC) value. Oxacillin and amoxicillin were tested as the positive controls against *S. aureus* and *E. coli*, respectively and ethanol was tested as the negative control. The test was carried out in triplicate.

## RESULTS AND DISCUSSION

**Isolation of *β*-sitosterol and synthesis of its derivatives**

*β*-Sitosterol is a secondary metabolite present in thousands of higher plants and has been isolated from various resources (Sayeed, 2016). In the present study, *β*-sitosterol was isolated from avocado fruit pulps. However, the extraction was very difficult as it contains a relatively large amount of water and hence the fruit pulp was freeze-dried first and then, the residue was used for the extraction with DCM. Further, the dry loading method was applied in column separation of *β*-sitosterol from the crude oil extract since the crude oil had an affinity towards the glass walls. Avocado is quite rich in *β*-sitosterol and therefore, compound 1 was isolated with a yield of 0.28%. The compound isolated was compared with the authentic sample using co-TLC elution with three different solvent systems and further confirmed with the spectroscopic data analysis. The important structural functionalities responsible for the aforementioned bioactivities of *β*-sitosterol are C5-C6 olefin and C3 hydroxyl group in addition to the tetracyclic core structure. Synthesis of derivatives was mainly designed by modifying those functional groups.




Reagents and conditions : (a)*m*-CPBA, DCM at °C, (b)Pd/C, $H_2$, MeOH, rt.
(c)Acetic anhydride, Pyridine, rt. (d)1)9-BBN, THF 2)$H_2O_2$, NaOH

**Figure 2:** Semisynthetic derivatives of *β*-sitosterol, Compound 2-5.

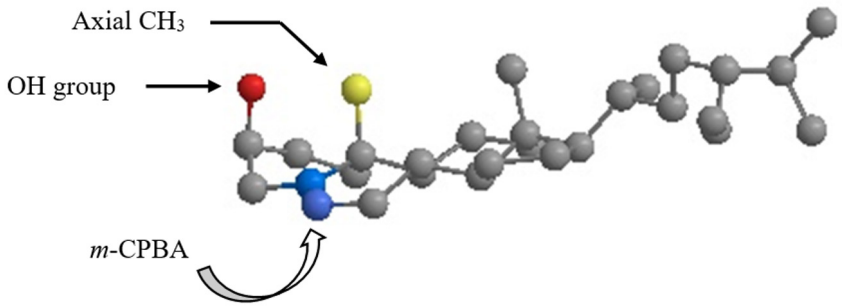



**Figure 3:** Attack of *m*-CPBA from the Si phase.

Figure 2 showed the schematic diagram with the reaction conditions used in the synthesis of semisynthetic derivatives of *β*-sitosterol (compounds 2-5). The 5-epoxy *β*-sitosterol (compound 2) was prepared by Prilezhaev reaction using *m*-CPBA and the product was obtained in 93% yield. Even though two stereoisomers of epoxide (*α* and *β*) were expected (Rega *et al*., 2007), the TLC pattern of the product mixture showed a single spot even in different solvent systems, which indicates the formation of one product. A similar study related to cholesterol, reported by Poirot and Silvente-Poirot explained that the *α* and *β* epoxides of cholesterol could be easily identified based on the $^1$H NMR spectral signals of H6. The α epoxide has a H6 proton signal around 2.75 - 2.95 ppm while the *β* epoxide has a H6 proton signal around 3.00 - 3.15 ppm. Since the H6 proton signal of compound 2 has a ppm value of 2.9, it evidenced the formation of the product, *α* epoxide of *β*-sitosterol (Poirot and Silvente-Poirot, 2013). Further, *α* epoxide formation would be the favoured product due to the steric hindrance caused by axial methyl group and hydrogens in adjacent carbons on the Re phase which facilitate the attack of *m*-CPBA from the Si phase (Figure 3) (Elgendy *et al*., 2007).

The disappearance of the C6 olefinic proton peak at 5.33 ppm and the appearance of a new doublet at 2.91 ppm in the resulting $^1$H-NMR spectrum confirmed the formation of the epoxide. In addition, the disappearance of the C=C bond stretching peak at 1654 cm$^{-1}$ in the IR spectrum also supported the formation of the desired product.

*β*-Sitosterol was subjected to hydrogenation over Pd/C in methanol at room temperature under a hydrogen atmosphere to yield compound 3 in 96% yield. Augustine and Reardon report that Pd is the best catalyst to perform hydrogenation of sterols due to its reactivity and ability to give the pure isomeric product (Augustine and Reardon, 1969). Spectral signals of this compound showed the absence of peaks that arise due to the C6 olefinic proton in $^1$H-NMR at 5.33 ppm and C=C stretching peaks at 1654 cm$^{-1}$ in IR confirmed loss of unsaturation during the reaction.

The acyl ester of *β*-sitosterol (compound 4) was synthesized upon reaction with acetic anhydride in pyridine in a 96% yield. Distillation of pyridine over KOH resulted in a higher yield and shorter reaction time. The $^1$H-NMR spectrum of product 4 clearly showed the upper field shift of the C3 proton peak from 3.51 to 4.62 ppm due to the substitution of an acyl group. In addition, the broad peak around 3400 cm$^{-1}$, which is responsible for the C3 hydroxyl group was absent in the IR spectrum and a new peak at 1715 cm$^{-1}$ due to the C=O stretching of acyl ester was observed.

Anti-Markovnikov hydration was carried out to add a hydroxyl functional group (-OH) to the C6 position using

**Table 1:** Minimum Inhibitory Concentration values of *β*-sitosterol and its derivatives.

| Bacterial Strain | MIC values of the compounds (ppm) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | PC | NC |
| ***S. aureus*** | 512 | 512 | > 1024 | > 1024 | 512 | < 1[a] | > 1024 |
| ***E. coli*** | 512 | 512 | > 1024 | > 1024 | 512 | 4[b] | > 1024 |

A - Oxacillin; b - Amoxicillin; PC – Positive Control; NC – Negative Control.

the two-step method, hydroboration followed by oxidation, and compound 5 has resulted in a 70% yield. In the present study, only the 6α product was obtained since only the Si face attack of 9-BBN is feasible due to the steric hindrance of the methyl group on Re face and the bulky nature of the 9-BBN. Bracher and Litz reported that the desired product could be achieved in high regio-selectivity in good yield with the use of 9-BBN due to its bulky nature (Bracher and Litz, 1996). The $^{1}$H-NMR spectrum showed the peak at 3.85 ppm in addition to the C3 proton peak at 3.82 ppm which indicates the presence of another carbinol proton at C6 where the new hydroxyl group was introduced. Additionally the absence of corresponding peaks for C6 olefinic proton in $^{1}$H-NMR between 4 ppm and 6 ppm and C=C stretching peaks around 1450 cm$^{-1}$ in IR spectra, also confirmed the formation of C6-hydroxyl.

Although derivatives of *β*-sitosterol such as long-chain esters (Farshori *et al*., 2009) and epoxide (Zhang *et al*., 2005) have been synthesized, the addition of a hydroxyl group at C6 position through anti-Markovnikov hydration has not been reported to the best of our knowledge.

### *In-vitro* antibacterial activity

*β*-Sitosterol and its semi-synthetic derivatives (compound 2-5) were subjected to *in vitro* antibacterial activity study against *S. aureus* ATCC 29213 and *E. coli* ATCC 25922 using micro broth dilution method according to the CLSI guidelines and results were summarized in Table 1. Antibacterial activity was measured in terms of minimum inhibition concentration (MIC) which is the lowest concentration of an antibiotic needed to inhibit the visible growth of a microorganism after overnight incubation.

*In vitro* antibacterial susceptibility, study results revealed that the starting material, *β*-sitosterol, and the semi synthesized products 2 and 5 obtained from epoxidation and anti-Markovnikov hydration, respectively have a MIC value of 512 ppm against both Gram-positive and Gram-negative organisms. However, MIC values of products 3 and 4 obtained from hydrogenation and acetylation were higher than 1024 ppm.

Different research groups reported that the MIC value of *β*-sitosterol is less than 100 ppm against *S. aureus* (Sanches *et al*., 2005; Sen *et al*., 2012). In contrast, several research groups have reported a higher MIC value (1000 ppm) of *β*-sitosterol against *S. aureus* (Chandramu *et al*., 2003; Li *et al*., 2008; Kongkathip *et al*., 2009). However, our screening study showed that the MIC value for *β*-sitosterol using the micro broth dilution method is 512 ppm for both organisms.

Based on our results, hydrogenation of the double bond at C5-C6 has decreased the antibacterial activity while the epoxidation and addition of a hydroxyl group at C6 was able to preserve its bioactivity. Acetylation at the C3 position has also reduced the antibacterial activity compared to the parent compound. Solubility of a drug substance is one of the parameters that decisively influence the therapeutic effects as it needs to be well balanced with sufficient hydrophilicity to be soluble in aqueous biological liquids and lipophilicity to penetrate through biological membranes (Mattson *et al*., 1977). Therefore decreased antibacterial activity of compounds 3 and 4 due to hydrogenation and acetylation may be due to the decrease of solubility as they reduce the polarity and increase the bulky nature compared to the parent compound in the biological system. Similar results have been reported by Silva *et al.* (2012) where they observed a reduction of antibacterial activity upon esterification of free hydroxyl at C3 in ursolic and oleanolic acids. However, incorporation of heteroatom to the ring system via epoxidation and hydroboration/ oxidation has no reduction of the activity though it removed the C5-C6 olefin. That could be since the parent molecule is highly lipophilic in nature and hence increase of hydrophilicity has no negative impact. These findings showed that hydroxyl functionally at C3 is essential for the activity; however C3 OH itself is not sufficient and hence it could be envisioned that the pharmacophore may consist of either full or partial tetracyclic ring with some kind of interaction through C5-C6 olefin, C5-C6 epoxide, and C6-hydroxyl.

## CONCLUSION

*β*-Sitosterol has moderate antibacterial activity and was easily isolated from avocado fruit pulps with a 2.3% yield. Among the four derivatives synthesized via structural modifications of two functional groups, the compound with C6 hydroxyl of *β*-sitosterol was reported for the first time. According to the *in-vitro* antibacterial studies introduction of the epoxide ring system to the C5-C6 bond or a hydroxyl group at the C6 position has not decreased the bioactivity while removal of the C5-C6 double bond and protection of the C3 hydroxyl group has reduced the activity. Hence, it can be concluded that the presence of C5-C6 olefin, C5-C6 epoxide, and C6 hydroxyl along with the hydroxyl group at the C3 position has a great influence on its antibacterial activity.

## ACKNOWLEDGEMENT

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## DECLARATION OF CONFLICT OF INTEREST

The authors declare that no conflicts of interest.

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