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Synthesis and antibacterial activity of epoxide from hyptolide (*Hyptis pectinata* (L.) Poit) against Gram-positive and Gram-negative bacteria

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ABSTRACT

Recently, drug resistance due to excessive use of antibiotics has become a severe problem, and alternative antibiotics' development has become an urgent problem. Epoxy hyptolide is a compound of the synthesis of hyptolide through the reaction using meta-chloroperoxybenzoic acid (mCPBA) oxidizer, potentially developed as a natural antibiotic. This study aimed to investigate the relationship between hyptolide and its epoxide structure on the antibacterial activity against Gram-positive and Gram-negative bacteria. The results indicated that epoxy hyptolide was a successful synthesis of hyptolide from the isolation of the leaves of *Hyptis pectinata* (L.) Poit using the mCPBA. The chemical characterization of hyptolide and its epoxidation revealed a melting point of 86.9°C–87.8°C and 79°C–80°C, respectively. The Fourier-transform infrared spectrum of epoxy hyptolide showed the presence of a lactone ring on wavenumbers 1,250 cm^{-1} and 814 cm^{-1}. On the other hand, a detailed ^1H nuclear magnetic resonance spectrum of a chemical shift of 3.5 ppm indicated the presence of an oxygen ring due to the transformation of C=C olefin double bond into an epoxide form. Furthermore, the test of the antibacterial activity of hyptolide and epoxy hyptolide was carried out by disk diffusion method. The results revealed that hyptolide and epoxy hyptolide have a border antimicrobial spectrum in a dose-dependent manner. The maximum inhibition zone (IZ) of hyptolide was observed in *Bacillus subtilis* and the IZ was found to be 28.00 mm in comparison with amoxicillin as the control had 7.58 mm IZ. Interestingly, the most effective antibacterial activity in *Salmonella typhi* caused by the presence of epoxy hyptolide with the maximum IZ was 21.80 mm, compared to amoxicillin that had 6.320 mm IZ. It can be concluded that Gram-negative bacteria was more susceptible to epoxy hyptolide in comparison to hyptolide. The cell wall structure of the Gram-positive and Gram-negative bacteria could be the main reason for the bacteria's susceptibility.

INTRODUCTION

Hyptis pectinata (L.) Poit is a family Lamiaceae plant that can be found in tropical areas such as Brazil, Mexico, India, and Indonesia. This plant popularly known in northeast Brazil as "sambacaita" or "canudinho" is an aromatic herbaceous plant clustered in axillary inflorescences with small bilabial flowers (Basílio *et al*., 2006; Franzotti *et al*., 2001). In Indonesia, *Hyptis pectinata* has not been cultivated, but it grows wild and is underutilized by the community (Suzery *et al*., 2012). The plant has a lot of chemical compounds and biological activities as reported earlier (Luzuriaga-Quichimbo *et al*., 2018; Suzery *et al*., 2020). The phytochemical content of *Hyptis pectinata* generally has a skeleton of α, β-unsaturated lactones, such as hyptolide compounds (Achmad *et al*., 1987), pectinolides A-C, sambacaitaric acid, rosmarinic acid (Falcao *et al*., 2013), pectinolides D-G (Boalino *et al*., 2003), and spicigerolide (Almtorp *et al*., 1991). The discovery of α, β-unsaturated lactones is an interesting study because of its potential effect as antinociceptive, antiedematogenic (Franzotti *et al*., 2001), antileishmanial (Falcao *et al*., 2013), antimalarial (Melo *et al*., 2006), antibacterial (Santos *et al*., 2008), and anticancer (Asy

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and Suzery, 2019; Amalina *et al*., 2020; Suzery *et al*., 2020). The previous study reported that hyptolide has a basic framework of α, β-unsaturated C12-δ-lactones with a chemical formula, $C_{18}H_{24}O_8$ (Achmad *et al*., 1987). The structure has a center of action in the isolated olefin group. The transformation of an isolated olefin group contained in hyptolide (C6–C7) was successfully carried out by our research group through the reaction using meta-chloroperoxybenzoic acid (mCPBA) oxidizer. Unfortunately, the activity of the transformation structure of hyptolide has never been explored, whereas previous research explained that the functional group has a crucial role in the activity. Among these properties are antimicrobial activities exhibited by the epoxide structure of the compound (Husain and Shaharyar, 2015). Erythromycin is modified to be 10, 11-epoxy acylide erythromycin which is shown to increase its antibacterial activity (Nie *et al*., 2013). The study supported the previous research results that epoxides derived from chalcones increase antibacterial activity against Gram-positive and Gram-negative bacteria (Husain and Shaharyar, 2015). Other studies also explained that the oxirane ring in the epoxide structure has an important role as antibacterial (Thirunarayanan and Vanangamudi, 2016). Based on these data, in this study, we evaluated the relationship between the structure of hyptolide and its transformation of epoxy hyptolide in the antimicrobial activity against Gram-positive and Gram-negative bacteria.

MATERIAL AND METHODS

Plant material

The herbs of *Hyptis pectinata* were collected from the local area East Kanayakan Dago, Bandung, Indonesia (latitude −7.0460282; longitude 107.7915393). The plant material was classified and authenticated by a biologist at the Ecology and Biosystematics Laboratory, Faculty Science and Mathematics, Diponegoro University, Indonesia. A reference specimen of the plant is deposited with a voucher number of MS 100562 at the Herbarium Biology, Faculty Science and Mathematics, Diponegoro University, Indonesia.

Isolation and purification of hyptolide

The extraction process of the dried *Hyptis pectinata* (L.) Poit was carried out using percolation and isolation of the hyptolide crystal by fractionations, followed by the literature procedure (Achmad *et al*., 1987; Meiny *et al*., 2012). After isolation, the purification of hyptolide crystals was carried out by the recrystallization method according to a previous research study with slight modification (Suzery *et al*., 2019). Recrystallization methods use ether to dissolve the hyptolide. Then, the filtrate was filtered using a Buchner funnel to obtain pure hyptolide crystals. The hyptolide crystals analyzed its purity through melting point test and chromatography using silica gel G60 F254 thin-layer chromatography (TLC) in 60% ether/40% chloroform and tested antibacterial activity against five types of bacteria.

Synthesis and characterization of epoxy hyptolide

Epoxidation procedures were prepared according to our previous research protocols (Suzery, 1989) with some modifications according to Bradley *et al*. (1997) and Hussain *et al*. (2014). It was prepared by mixing the hyptolide (368 mg), which is weighed into a 100 ml round bottom flask, with $CHCl_3$ (15 ml), benzoic acid (C_6H_5COOH 345 mg), mCPBA (20 mg), and a magnetic stirrer which are introduced into the flask and the mixture is stirred and cooled at 0°C for 24 hours. Furthermore, determining the end of the epoxidation reaction was confirmed by the similarity of retention time value using TLC. The resultant product was purified using the recrystallization method as described previously.Identification of the crystals was obtained using nuclear magnetic resonance (NMR) and Fourier-transform infrared (FTIR) according to Ahmad *et al*. (2014) and Suzery *et al*. (2019). NMR spectrometry was carried out by using Nanalysis-type ready NMR, Canada. ^{1}H spectra were measured at 500 MHz. Approximately, 2 ml of each sample was dissolved in 2 ml of nondeuterated chloroform ($CDCl_3$) (Sigma-Aldrich-288306), placed in the NMR tube to produce a sample depth of approximately 3.5 to 4 cm^{-1} and ready for NMR spectrometer analysis. The description of the type of ^{1}HNMR detected was based on the characteristic NMR absorption table published in Organic Chemistry (Janice, 2008).

Furthermore, to prove the presence of an oxirane ring function group as a marker of epoxide formation by using FTIR (Perkin Elmer), the crystalline samples were introduced directly into FTIR. The scan range employed was from 400 to 4,000 cm^{-1} with a resolution of 4 cm^{-1}. The chemical bond in a molecule was detected by interpreting the infrared transmittance spectrum. The identification of the functional group in the compound was based on the table of characteristic IR absorptions published in Organic Chemistry (Janice, 2008).

Bacterial and growth conditions

The bacterial spp. used for the test were Gram-positive: *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* FNCC-0047, and *Streptococcus mutants* ATCC 26176, and gram-negative*: Escherichia coli* ATCC 35218 and *Salmonella typhi* ATCC 19430. All the stock cultures were collected from the integrated laboratory unit, Diponegoro University, Semarang, Indonesia. All the bacterial strains used in this study were maintained by sub-culturing them for 24–48 hours at 37°C on the nutrient agar/broth (Merck).

Antibacterial activity assay

The extract, hyptolide, and epoxy hyptolide were screened for their antibacterial activity in comparison with the standard antibiotic amoxicillin (25 mg/ml) by disk diffusion method according to Mama *et al.* (2019) and Razmavar *et al.* (2014) using *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus mutants*, *Escherichia coli*, and *Salmonella thyposa* as test organisms. This method possesses the potential to see growth inside of the plate and the colony can be visualized in the inhibitory zone (Sachdeva *et al*., 2015).

Bacterial suspension of 0.5 McFarland (1.5×10^8 colony forming unit CFU/ml) was obtained from all the bacterial strains and then, surface culture was carried out by spreading with a sterile cotton swab on Mueller Hinton agar (Merck). Each sample was individually loaded on the 6 mm sterile disk at concentrations of 25, 50, and 75 mg/ml and subjected to antibacterial activity. For negative control, the disks were loaded with 10% dimethyl sulfoxide (Sigma-Aldrich). After diffusion of the sample into the medium, the plates were incubated for 24 hours at a temperature

of 37°C. After incubation, antibacterial activity was evaluated by measuring the diameter of the inhibition zone (IZ) around the disks using a caliper. The assay was repeated thrice. Antibacterial activity was expressed as the mean zone of inhibition diameters (mm) produced by each sample. The diameter of the IZ was considered resistant or not sensitive (8.0 mm), moderately sensitive (8.0–14.0 mm), relatively sensitive (14–20 mm), and extremely sensitive by more than 20 mm (Singh Arora *et al*., 2009).

Statistical analysis

The results were expressed as mean ± SEM. Statistical analysis of the data was carried out using one-way ANOVA and the results were considered significant when $p < 0.05$.

RESULTS AND DISCUSSION

Antibiotic resistance is harmful to human health because most infectious organisms recognize and establish a tolerance for the mechanism of drug action (Peterson and Kaur, 2018; Zhang *et al*., 2018). Because of frequent resistance development and the potentially harmful effect on the use of conventional antibacterial drugs (Founou *et al*., 2017; Matsunaga and Hayakawa, 2018), there is continuous research to explore new natural antibacterial agents to reduce resistance side effects and improve efficacy against infectious microorganisms (Andrade *et al*., 2017). *Hyptis pectinata* extract is one of the natural agents which can potentially be developed as an anticancer, antioxidant, and antibacterial (Amalina *et al*., 2020; Santana *et al*., 2019; Suzery *et al*., 2020). Nevertheless, tests of the antibacterial activity of hyptolide have never been attempted. In comparison, the epoxy ring was found to be the polar form of the nature of carbonyl group responsible for the medical activities. Some natural epoxide demonstrated biological activities (Marco-Contelles *et al.*, 2004). Generally, epoxide possesses many biological activities, such as antianalgesic activity (Inceoglu *et al*., 2008), anti-inflammation (Morisseau *et al*., 2012), cytotoxicity (Ye *et al*., 2002), and tumorigenicity (Pal *et al*., 2013). Hence, the purpose of this research was to evaluate the relationship between the structure of hyptolide and its epoxy hyptolide transformation and the antibacterial activity of Gram-positive and Gram-negative bacteria strains.

Hyptolide isolation and purification

First, we isolated the hyptolide compound from *Hyptis pectinata* (L.) Poit methanolic extract using recrystallization methods. The hyptolide compound (yellowish) was obtained as much as 2.78 g (0.83%) from 335 g of leaves of dried *Hyptis pectinata* (L.) Poit. Hyptolide yields generally ranged from 0.30% to 0.94% (Achmad *et al*., 1987). The yield is much smaller than a previous research study which reported that the yield of isolation of the hyptolide compound from *Hyptis pectinata* (L.) Poit was 2.00% (Gorter, 1920). The percentage difference is due to the crop-taking season and geographical factors that have an important effect on the percentage of yield obtained (Agnolucci and De Lipsis, 2019). Thus, we determine the melting point to analyze the purity of hyptolide; the results showed that the hyptolide crystals have a melting point of 86.9°C–87.7°C. A narrow melting point range (0.5°C–2°C) usually indicates that the substance is fairly pure. These results are not much different from the results reported by previous research studies, like 88.5°C (Gorter, 1920), 88.5°C (Birch and Butler, 1964), 87°C–88°C (Achmad *et al*., 1987), and 87°C–88°C (Suzery, 1989). Furthermore, to confirm its purity, spot analysis was carried out by TLC with various mobile phases. The hyptolide compound gives a single brown spot with a retention factor (*Rf*) value between 0.45 and 0.65 (Table 1). Based on these results, it can be concluded that the hyptolide crystal isolated from *Hyptis pectinata* extract is a pure compound, causing the fairly pure crystalline compounds to usually have the smallest range (0.5°C–2°C), a sharp melting point (Klepper, 2009), and a single spot on TLC analysis (Marston, 2011).

Hyptolide transformation using mCPBA reaction and its characterization

Hyptolide epoxidation reaction approaches are based on the method developed by Bradley *et al*. (1997) and Hussain *et al*. (2014), with slight modification (Fig. 1). The TLC analysis has a retention factor (*Rf*) value of 0.44 as a single soft brown zone (A) and 0.32 as a single black zone (B) on the TLC chromatogram showed the hyptolide and epoxy hyptolide separation, respectively (Fig. 2). The existence of a single-spot retention factor (*Rf*) showed that the compound of isolation was perfectly pure of hyptolide and its epoxide. Based on the different R*f* values between the two spots, it can be concluded that the spots are different compounds. After purification under the recrystallization method, we confirmed the purity of two compounds using a melting point assay. The purity assessment is the key parameter and the most critical in the case of a novel compound to which a biological is ascribed (Pauli *et al*., 2014). Our results showed that epoxy hyptolide has a melting point of 79°C–80°C which is different from the hyptolide melting point of 86.9°C–87.7°C. These results indicated that the epoxy hyptolide was successfully formed and had different characteristics from the previous compound. On the other hand, a small range of melting points (0.5°C–2°C) means that the material is relatively pure (Klepper, 2009).

NMR analysis of hyptolide and its epoxide chemical structure was further elucidated. The ^{1}H NMR analysis showed the same results as the previous studies (Sabitha *et al*., 2014). The ^{1}H NMR spectrum of epoxy hyptolide is shown in Table 2. The ^{1}H NMR spectrum and chemical shift of epoxide compounds indicated that the epoxy hyptolide compound had been formed. It is shown by the presence of the hydrogen spectrum corresponding to the composition of the epoxy hyptolide proton. The specific NMR spectrum shows that epoxy hyptolide is formed in the presence of three proton spectra with singlet multiplicity, and ^{3}H integration with a chemical shift of about 2.00 ppm which shows that the methyl group is attached to carbon atoms C-19, C-23, and C-27. Meanwhile, the proton on the C-14 atom is shown by the three integrated doublet peaks in the chemical shift of about 1.21 ppm. The specific spectrum of epoxy hyptolide is shown at 5.10 ppm with the integration of two protons which is the proton spectrum at C-2 and C-10. The C-2 and C-10 spectra produced are the same due to the same chemical environment. Atoms C-2 and C-10 are both directly bonded to oxygen atoms which are electronegative so that the resulting spectrum peaks are in the deshielding region (5.10 ppm). The spectral peaks of C-8 and C-9 which are carbon atoms in cyclic epoxide are shown in relatively close chemical shifts, namely 3.20 and 3.26 ppm, with double doublet multiplicity (dd). The C-5 and C-6 spectra are in the deshielding area, namely

Table 1. TLC analysis of hyptolide.

Mobile phase	Retention factor R_f
Ether	0.55
Chloroform	0.65
Benzena	–
Methanol	0.60
Ether : chloroform (3 :2)	0.45

The stain detection is carried out with 254 UV lamps and spraying with dilute sulfuric acid.

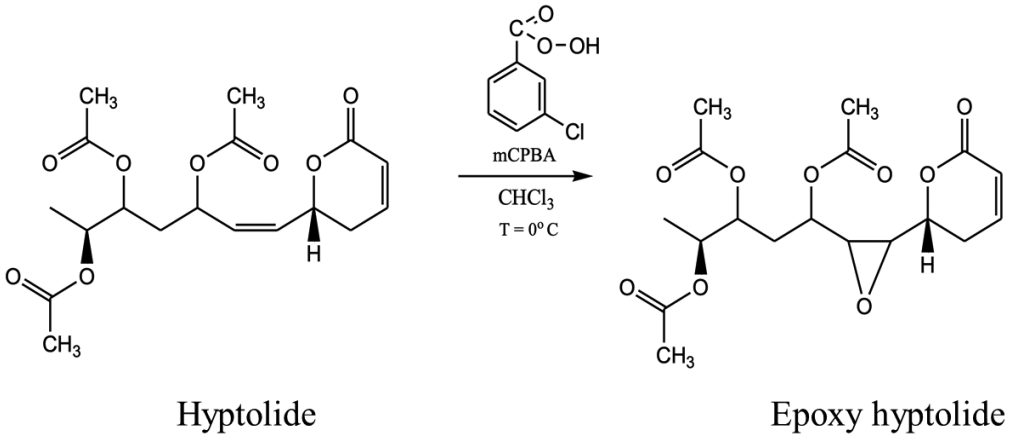

Figure 1. Scheme showing the reaction between epoxy hyptolide and hyptolide epoxidation with a ratio of 1 mol of hyptolide and 2 mol of mCPBA using 0.736 grams of hyptolide crystal dissolved into 15 ml of chloroform and 0.690 g of mCPBA as a reactant for 24 hours.

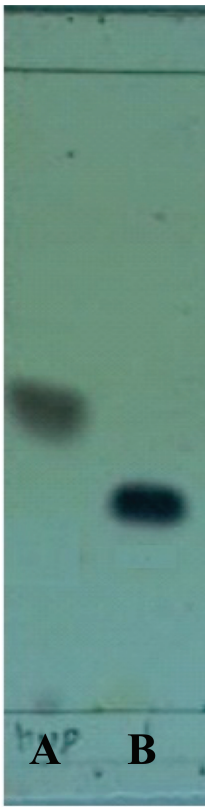

Figure 2. TLC separation of the hyptolide and epoxy hyptolide on silica gel 60 F254. Mobile phase using chloroform :ether (10:1). Sample application of 10 µl: (A) hyptolide and (B) epoxy hyptolide reaction products.

6.06 and 6.87 ppm, due to the position of the C-5 and C-6 atoms double bond (pi-bonding interaction), and are in the ring system resulting in chemical shear in the deshielding area. The peak of the spectrum at 5.00 ppm chemical shift belongs to the proton on the C-12 atom which is directly attached to the oxygen atom so that the spectrum is also in the deshielding region. The C-12 spectrum has triplet multiplicity because it is adjacent to three protons, namely two protons in the C-11 atom and one proton in C-13. The C-13 spectrum which is directly bound to oxygen atoms causing a peak to appear in the chemical shear deshielding is also at 4.61 ppm with multiplet multiplicity (m) because it is adjacent to three protons at C-14 and one proton at C-12 (Fig. 3).

On the other hand, we also evaluated using fingerprint analytical technique of FTIR spectroscopy to confirm the function group of epoxy hyptolide. Figure 4 shows epoxy hyptolide FTIR spectra at midinfrared region corresponding to wavenumbers of 4,000–400 cm^{-1} along with prominent peaks due to infrared transmission. Table 3 shows the compilation of the functional groups responsible for infrared transmission which may be correlated with the chemical compound of epoxy hyptolide. The IR spectrum of epoxy hyptolide showed the absorption of epoxide wavenumbers at 750–840 and 1,250 cm^{-1}, which indicate the presence of stretching C–O epoxide. The stretching is supported by the presence of C–H epoxide absorption at 2,990–3,050 cm^{-1}. In addition, the absorption of lactone at 1,735–1,750 cm^{-1} shows the stretching C=O lactone or ester which is supported by the absorption of C–O lactone and ester at 1,000–1,300 cm^{-1}. Interestingly, no stretching vibration of C=C in the epoxy hyptolide product indicates that the infrared spectrum of the product is different from pure hyptolide. After confirming the structure and functional groups, the antibacterial activity was analyzed using disk diffusion methods.

Antibacterial activity of hyptolide on Gram-positive and Gram-negative bacteria

This study shows antibacterial activity against clinically important Gram-positive and Gram-negative strains of a bacterial pathogen. The natural antibacterial agent found to be inhibitory to a broad range of bacteria has been reproducibly demonstrated at a high antagonistic capacity. In this investigation, we explored the effect of *Hyptis pectinata* extracts, hyptolide, and epoxy hyptolide on the antibacterial activity against Gram-positive and Gram-negative bacteria strains, *Staphylococcus aureus*, *Streptococcus mutants*, and *Bacillus subtilis,* as a Gram-positive bacterium. The presence of an IZ around the disks has determined the antibacterial activity. The concentration of the *Hyptis pectinata* extract, hyptolide, and epoxy hyptolide tested, ranging from 25 to 75 mg/ml, showed significant antibacterial activity against bacterial strains compared with amoxicillin (25 mg/ml) as a positive control.The high dose of a hyptolide revealed the maximum activity (28.00 ± 0.42 IZ) against Gram-positive *Bacillus subtilis* bacteria, followed by *Streptococcus mutants* (20.00 ± 0.28 IZ) and *Staphylococcus aureus* (15.50 ± 0.92 IZ) (Fig. 5A and B). Interestingly, the presence of *Hyptis pectinata* extracts, hyptolide, and epoxy hyptolide on the medium for 24 hours highly increased an IZ and exhibited strong antibacterial activity in a dose-dependent manner. The *Hyptis pectinata* extracts, hyptolide, and epoxy hyptolide showed activity against all the three types of bacteria. The diameter of the IZ produced was sensitive in *Bacillus subtilis* due to the addition of those samples. But interestingly, the strongest activity to *Bacillus subtilis* was obtained by the hyptolide compared to *Hyptis pectinata* extract and epoxy hyptolide. The presence of hyptolide may be responsible for the antibacterial activity. A glance at the active products listed in Figure 5A and B indicated that the presence of a,b-unsaturated

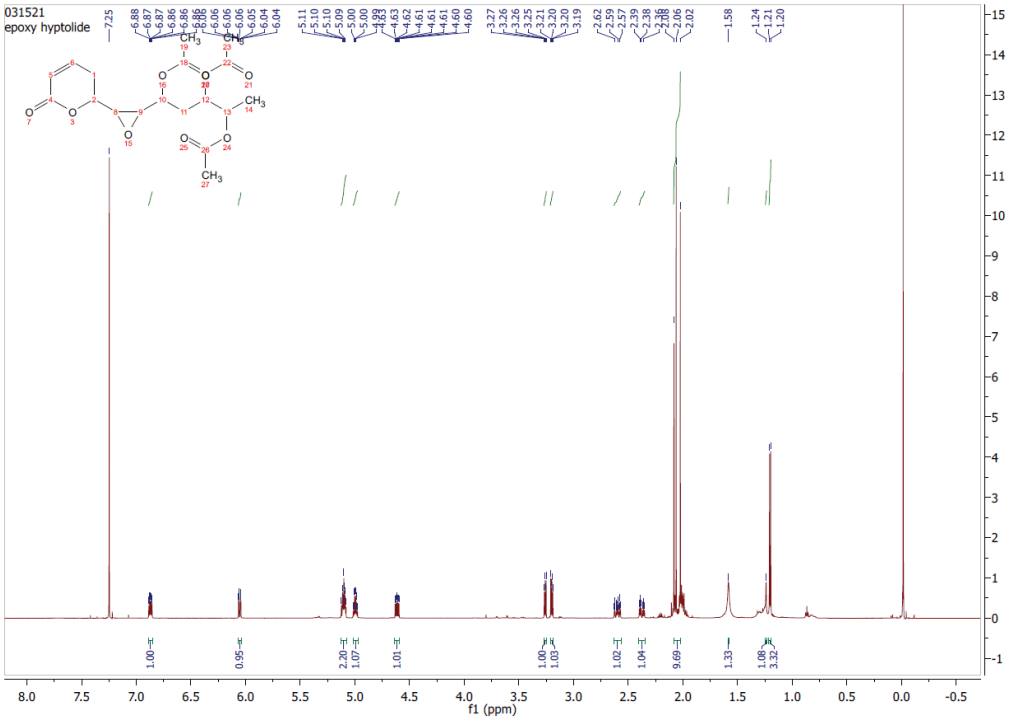

Figure 3. ^{1}H NMR spectrum of epoxy hyptolide (500 MHz, $CDCl_3$).

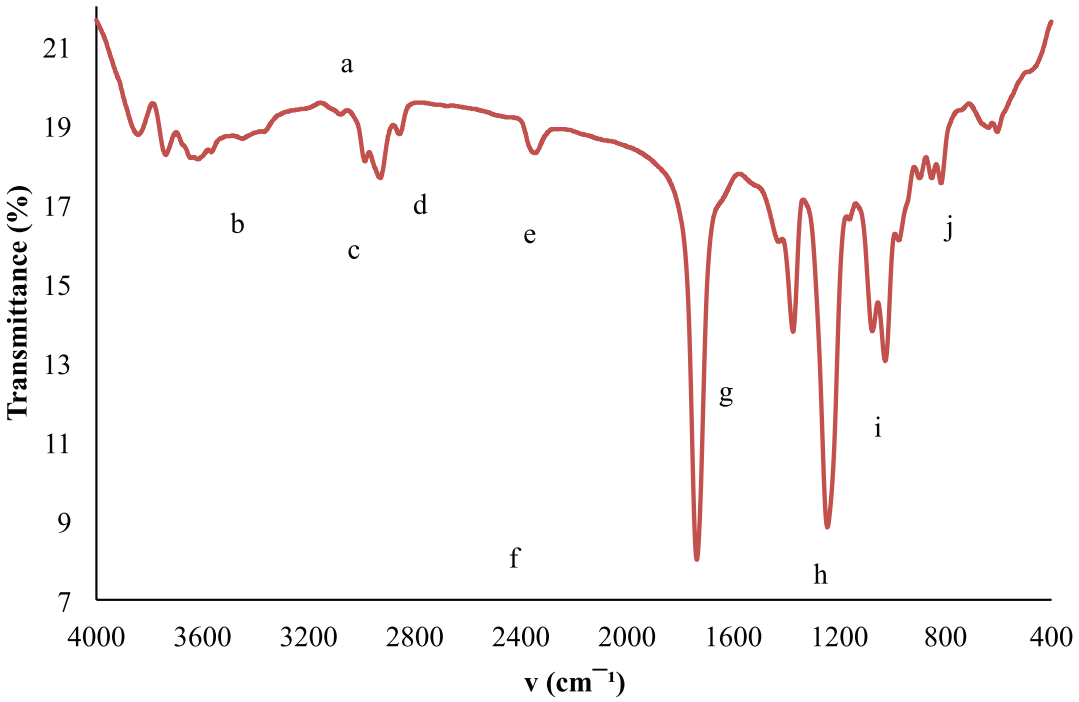

Figure 4. FTIR transmittance spectrum of epoxy hyptolide.

Table 2. ^{1}H NMR chemical shifts observed from the epoxidation of hyptolide.

Atomic C number	δH (ppm), multiplicity, integration
	Epoxy hyptolide
1	2.38; 2.59 (ddd, 1.02 H; 1.04H)
2,10	5.10 (m, 2.20H)
5	6.06 (dt, 0.95 H)
6	6.87 (ddd, 1.00 H)
8	3.20 (dd, 1.03 H)
9	3.26 (dd, 1.00 H)
11	1.58; 1.24 (dd, 1.33H; 1.08 H)
12	5.00 (dt, 1.07 H)
13	4.61 (m, 1.01 H)
14	1.21 (d, 3 H)
19	2.02 (s, 3 H)
23	2.06 (s, 3 H)
27	2.08 (s, 3 H)

Table 3. The functional groups are responsible for the absorption of epoxy hyptolide in the IR.

Assignment	Wavenumbers	Functional groups	
	Epoxy hyptolide	Hyptolide	
a	3,078 cm^{-1}	3,080 cm^{-1}	Stretching vibration of C-H sp2
b	2,992 cm^{-1}	–	Stretching vibration of C-H epoxide
c	2,938 cm^{-1}	2,960 cm^{-1}	Stretching vibration of C-H sp3 from metal
d	2,854 cm^{-1}	2,853 cm^{-1}	Stretching vibration of C-H sp3 from -CH2-
e	1,740 cm^{-1}	1,740 cm^{-1}	Stretching vibration of conjugated carbonyl (C=O) group
f	–	1,640 cm^{-1}	Stretching vibration of C=C not conjugated
g, i	1,377 and 1,033 cm^{-1}	1,225 and 1,170 cm^{-1}	Stretching vibration of C-O lactone and ester
h, j	1,250 and 814 cm^{-1}	-	Stretching vibration of C-O epoxide

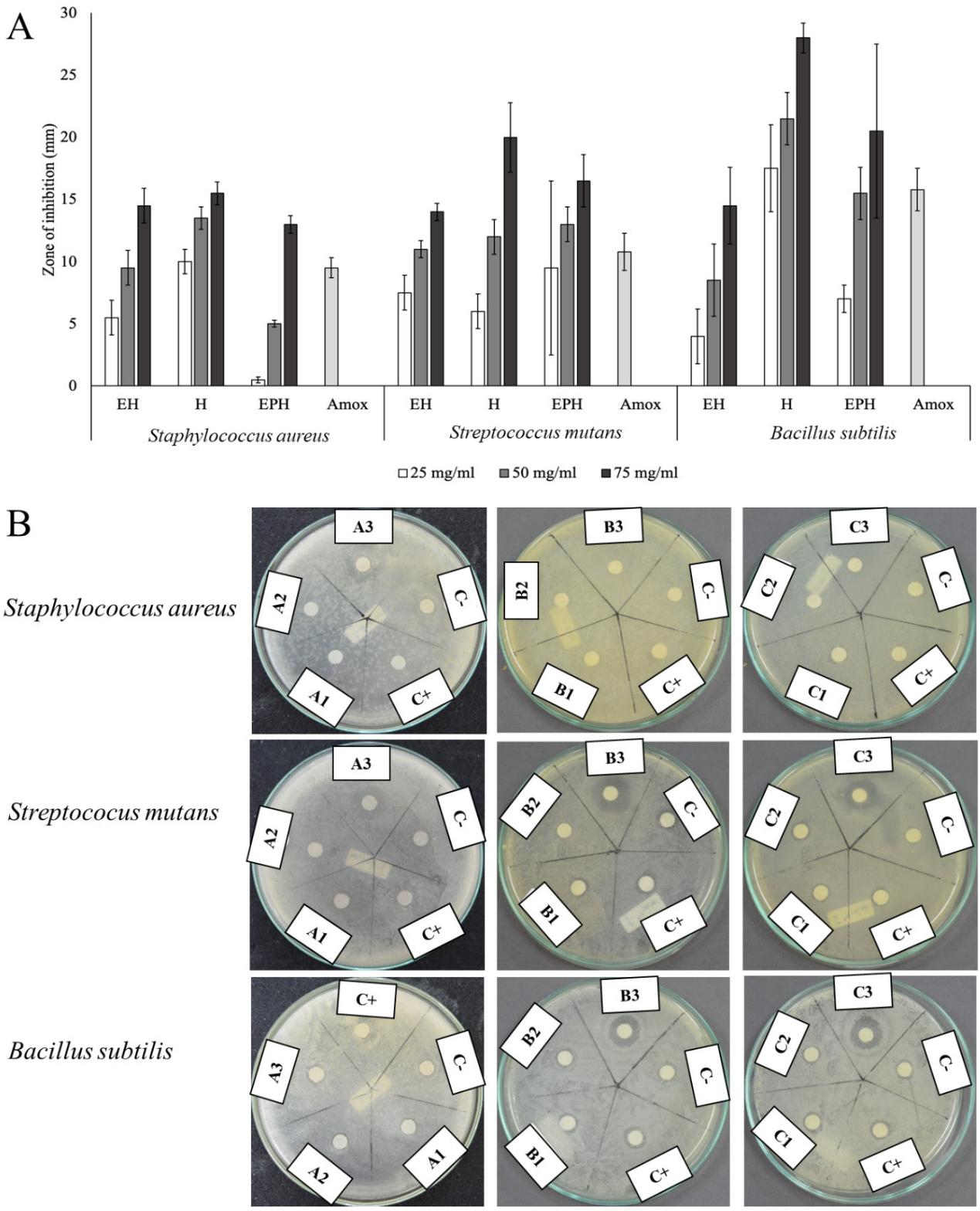

Figure 5. (A) Antibacterial activity of *Hyptis pectinata* methanolic extract, hyptolide, epoxide hyptolide, and amoxicillin against gram-positive organisms (mean ± SEM) (mm). Values are means of triplicate determination (n = 3) ± standard deviations. *EH = methanolic extract of *Hyptis pectinata*; H = hyptolide; EPH = epoxy hyptolide; Amox = Amoxicillin. (B) Morphological growth inhibition of extract, hyptolide, and epoxy hyptolide against gram-positive bacteria. A1: extract 25 mg/ml; A2: extract 50 mg/ml; A3: extract 75mg/ml; B1: hyptolide 25 mg/ml; B2: hyptolide 50 mg/ml; B3: hyptolide 75mg/ml; C1: epoxy hyptolide 25 mg/ml; C2: epoxy hyptolide 50mg/ml; C3: epoxy hyptolide 75 mg/ml; C+: amoxicillin; C-: control negative.

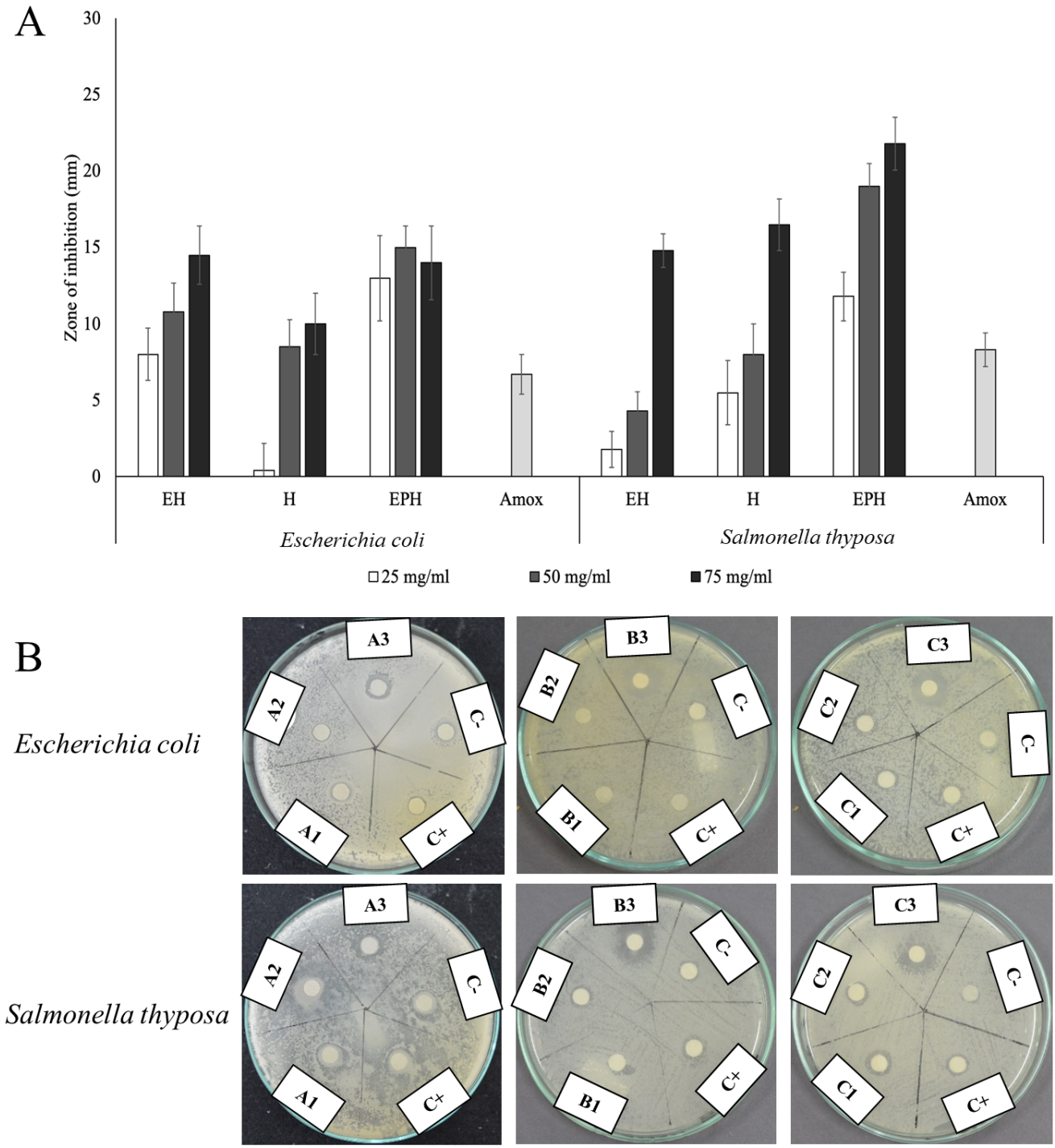

Figure 6. (A) Antibacterial activity of *Hyptis pectinata* methanolic extract, hyptolide, epoxide hyptolide, and amoxicillin against gram-negative organisms (mean ± SEM.) (mm). Value are means of triplicate determination (n = 3) ± standard deviations. *EH = methanolic extract of *Hyptis pectinata*; H = hyptolide; EPH = epoxy hyptolide; Amox = amoxicillin. (B) Morphological growth inhibition of extract, hyptolide, and epoxy hyptolide against gram-negative bacteria. A1: extract 25 mg/ml; A2: extract 50 mg/ml; A3: extract 75mg/ml; B1: hyptolide 25 mg/ml; B2: hyptolide 50 mg/ml; B3: hyptolide 75mg/ml; C1: epoxy hyptolide 25 mg/ml; C2: epoxy hyptolide 50mg/ml; C3: epoxy hyptolide 75 mg/ml; C+: amoxicillin; C-: control negative.

lactones in the molecule is necessary despite not being the only structural requirement for antibacterial activity (Cartagena and Bardon, 2008). In fact, the *Hyptis pectinata* extract is not more active than hyptolide. The non-effective antibacterial activity of the plant extract in this study can be attributed to the presence of various bioactive components that work antagonistically (Debalke *et al*., 2018). On the other hand, the *Hyptis pectinata* extract activity against Gram-positive bacteria indifferent with the findings of Miranda *et al*. (1993) and Santos *et al*. (2008) that obtain the minimum inhibitory concentration (MIC) of 12.5 µg/ml under

pectinolades A and C treatment (another secondary metabolite of *Hyptis pectinata* extract)

In the second set of experiments, the *Hyptis pectinata* extract, hyptolide, and epoxy hyptolide were tested against Gram-negative bacteria. *Escherichia coli* and *Salmonella thyposa* were used in the test. The maximum antibacterial activity on Gram-negative *Salmonella thyposa* (21.80 ± 0.74 IZ) is more potent than that of *Escherichia coli* (14.00 ± 0.42 IZ) due to the presence of a high concentration of epoxy hyptolide (Fig. 6A and B). The antibacterial activity on Gram-negative bacteria shows that the best inhibitory zone diameter results were obtained from the epoxy hyptolide compound in *Escherichia coli* and *Salmonella thyposa* bacteria compared with the results of the inhibitory zone of hyptolide and *Hyptis pectinata* extract. This means that the epoxy compounds are better used for Gram-negative bacteria than pure hyptolide and *Hyptis pectinata* extracts that contain various bioactive compounds. Interestingly, these results are different from those conducted by Miranda *et al*. (1993), on *Escherichia coli* Gram-negative bacteria using pure hyptolide compounds that do not produce inhibitory zones. The other reason may also be that the antibacterial activity increased due to the presence of epoxy structure; for Gram-negative bacteria, the epoxy structure individually showed more antibacterial activities than that of Gram-positive bacteria. This may be due to the presence of an extra outer membrane in Gram-negative bacteria, which consists of lipopolysaccharide and makes them permeable to lipophilic compounds, such as epoxy structure, whereas for Gram-positive bacteria, they have a peptidoglycan outer layer that is a hydrophilic barrier. It underlies the activity of hyptolide isolates more effectively because hyptolide is a polar compound. Even if the antibacterial activity of the plant extract was very low in this study, different researches revealed that the plant extract is more effective than the isolated compound of the extract.

In contrast to amoxicillin which showed lower activity in both Gram-positive and Gram-negative bacteria compared to the sample, amoxicillin has an IZ diameter of 7.58 and 6.69 against *Bacillus subtilis* and *Escherichia coli*, respectively. The difference in sensitivity between Gram-negative and Gram-positive bacteria may be due to the variability in the composition of their cell wall. The bacterial cell wall of Gram-positive bacteria consists of 100 layers of peptidoglycans. This is obviously an oversimplification as an explanation and other mechanisms are likely to play a part. Gram-negative bacteria resistance to b-lactam antibiotics, such as amoxicillin, originates from the lactamase enzyme being secreted in the periplasmic space between the thin outer membrane and the cytoplasmic membrane (Henley-Smith *et al*., 2014).

These results conclude that the difference in bacterial sensitivity to antibacterial is influenced by the structure of the bacterial cell wall. Gram-positive bacteria tend to be more sensitive to antibacterial because the structure of Gram-positive bacterial cell walls is simple, making it easier for antibacterial compounds to enter into Gram-positive bacterial cells. The underlying mechanisms of hyptolide on the inhibited bacterial growth need to be explored further.

CONCLUSION

Newly developed epoxy hyptolide compounds were prepared in good yield and characterized by various instrumental techniques. It has been observed that the presence of epoxy ring enhances antibacterial activity, especially on Gram-negative bacteria. More importantly, our findings suggest that epoxy hyptolide has the potential to be developed as a natural antibacterial agent with better effects and possibly fewer resistance side effects.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest relevant to the contents of this article.

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AUTHORS' CONTRIBUTIONS

The contributions of each author are stated as follows: BC and MZ contributed to the conception of the work; BC, MZ, NDA, and WW contributed to the acquisition of the work; NDA, BC and DNB contributed to the analysis and interpretation of data; NDA, BC, and MZ contributed to drafting the work; BC and MZ contributed to revising the work critically; BC was responsible for giving the final approval of the manuscript.

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