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ORIGINAL ARTICLE

Chemical constituents and bioactivities from the leaves of *Beilschmiedia glabra* Kosterm (Lauraceae)

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ABSTRACT

Two butanolides, subamolide D (**1**) and subamolide E (**2**), along with β-sitosterol (**3**), β-sitostenone (**4**), lupeol (**5**), taraxerol (**6**), and 24-methylenelanosta-7,9(11)-diene-3β,15α-diol (**7**) were isolated from the leaves of *Beilschmiedia glabra*. The structures of the isolated compounds were elucidated by extensive spectroscopic data analysis and comparison with respective literature data. The compounds were tested for DPPH• radical scavenging, antimicrobial, acetylcholinesterase and lipoxygenase inhibitory activities. Compounds **1** and **2** displayed the strongest lipoxygenase assay with an IC_{50} value of 5.1 and 5.5 μM, respectively. All compounds showed weak activity on DPPH• radical-scavenging, antimicrobial, and acetylcholinesterase inhibition assays.

Keywords: Lauraceae, *Beilschmiedia*, DPPH•, antimicrobial, acetylcholinesterase, lipoxygenase

INTRODUCTION

The pantropical genus *Beilschmiedia* Nees of the family Lauraceae comprises about 250 species, distributed mainly in tropical regions of Asia and Africa. Most of these species grow in tropical climate, and are widespread in tropical Asia, Africa, Australia, New Zealand, Central America, Carribean Islands and South America (1). A few species of this genus are used in traditional medicines for the treatment of various diseases. In Africa, several *Beilschmiedia* species have been used as remedies in folkloric medicine. *B. manii* is used for the treatment of dysentery and headache, and also as an appetite stimulant (2). In Cameroon, *B. anacardiodes* seeds are used as spice while the stem bark are used as to treat uterine tumors, rubella, female genital infections, and rheumatisms (3). Besides in the treatment of diarrhea, in Peninsular Malaysia, a decoction of bark of *B. pahangensis* is used as an imbibe after childbirth and to ease stomachache. In Madagascar, *B. cryptocaryoides* the fruit, bark, and leaf are utilized by the locals for treatment of contagious disease and malaria (4). This genus produces alkaloids, endiandric acid derivatives, essential oils, fatty acids, epoxyfuranoid lignans, flavonoids and terpenoids, and many of these compounds possess antioxidant, antibacterial, antimalarial and anti-tuberculosis activities (5-7).

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We have recently reported the phytochemical studies of the stem barks extracts of this species and managed to isolate two new phenanthrene alkaloids, beilschglabrines A and B (8). Beilschglabrine A displayed considerable activity in the acetylcholinesterase (IC_{50} 50.4 μM), the DPPH• radical scavenging (IC_{50} 115.9 μM) and the lipoxygenase (IC_{50} 32.8 μM) assays. In addition, we also managed to report the chemical compositions and biological activities of the leaf and bark oils from this species. The leaf and bark oil contained mainly β-eudesmol (15.4-19.3%) and β-selinene (12.2-16.9%). The leaf oil had the highest phenolic content (233.4 mg GA/g), while the bark oil showed potent activity in the β-carotene/linoleic acid bleaching assay (77.6%). Both the leaf and bark oils were active against *Candida glabrata* and *Saccharomyces cerevisiae* with MIC values of 31.3 and 62.5 μg/mL, respectively. Tyrosinase (leaf 73.7%; bark 76.0%) and acetylcholinesterase (leaf 48.1%; bark 45.2%) inhibitory activities, and anti-inflammatory (leaf 59.7%; bark 48.9%) property were also identified (9).

In continuation of our search for bioactive compounds, we have performed a phytochemical study on the leaves part of *B. glabra*, which has led to the isolation of two butanolides; subamolide D **1** and subamolide E **2** (10). In addition, five known triterpenes β-sitosterol **3** (11), β-sitostenone **4** (12), lupeol **5** (13), taraxerol **6** (14), and 24-methylenelanosta-7,9(11)-diene-3β,15α-diol **7** (15) were also isolated (**Figure 1**). This paper describes the structural elucidation of **1** and **2**, together with the DPPH• radical scavenging activity, antimicrobial, acetylcholinesterase and lipoxygenase inhibitory activities of the isolated compounds.

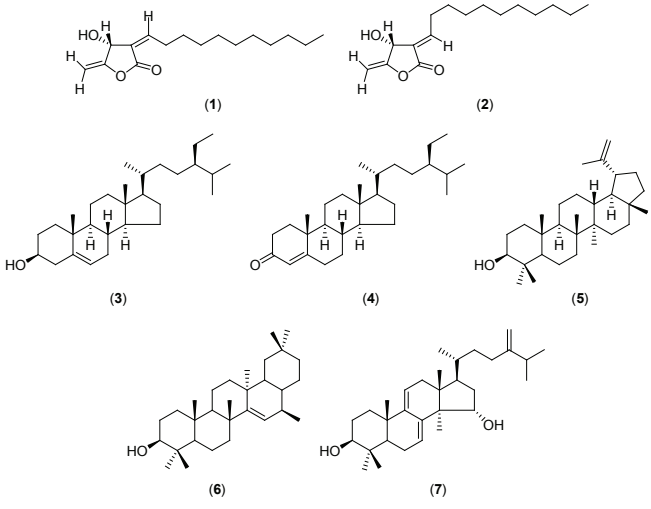

Figure 1. Chemical structures of compounds (**1**-**7**) isolated from the leaves of *B. glabra*

MATERIAL AND METHODS

General experimental procedures

The mass spectra were obtained on a Finnigan-MAT-95 mass spectrometer from National University of Singapore. The UV spectra were obtained in methanol on a Shimadzu UV 1601PC spectrophotometer. The IR spectra were obtained on a Perkin- Elmer 1600 FTIR spectrophotometer. The 1D and 2D NMR spectra were recorded in deuterated chloroform on a Bruker Avance 400 MHz spectrometer, chemical shifts (d) are reported in ppm on d scale, and the coupling constants (*J*) are given in Hz. Vacuum liquid chromatography (VLC) was performed using Merck silica gel 230-400 mesh, while column chromatography (CC) was performed using Merck silica gel 70-230 mesh. Preparative thin layer chromatography (PTLC) was prepared using silica gel 60 PF_{254}. Thin layer chromatography (TLC) aluminum sheets pre-coated with silica gel 60 F_{254} (0.2 mm thickness) was used to detect and monitor components presence in the crude samples or fractions. The TLC and PTLC spots were visualized under UV light (254 and 366 nm) followed by spraying with 5% H_2SO_4 and 1% vanillin in MeOH and heating at 120°C for 5 min. All solvents were AR grade.

Plant material

The leaves of *B. glabra* were collected from Kluang, Johor in October 2014. The species was identified by Dr. Shamsul Khamis from the Institute of Bioscience (IBS), UPM and the voucher specimen (SK2570/14) deposited at the Herbarium of IBS.

Extraction and isolation

Cold extraction of the powdered leaves (1 kg) of *B. glabra*, sequentially, with n-hexane, EtOAc and MeOH yielded the crude extracts, *n*-hexane (BGLH: 5.0 g, 0.50%), EtOAc (BGLE: 10.8 g, 1.08%) and MeOH (BGLM: 18.1 g, 1.81%). The *n*-hexane leaves extract (BGLH, 5.0 g) was fractionated by VLC and eluted with *n*-hexane:$CHCl_3$:EtOAc to afford eight fractions (BGLH 1-8). Fractions BGLH 3-7 was combined and purified by CC eluted with *n*-hexane:$CHCl_3$ followed by PTLC to yield subamolide D **1** and subamolide E **2**. The EtOAc extract (BGLE, 10.0 g) was fractionated by VLC and eluted with *n*-hexane:EtOAc to afford six fractions (BGLE 1-6). Fractions BGLE 2-4 were combined and subjected to CC followed by PTLC to afford β-sitosterol **3**, β-sitostenone **4**, and lupeol **5**. The MeOH extract (BGLM, 15.0 g) was

subjected to VLC and eluted with *n*-hexane:CHCl3:MeOH to yield twelve fractions (BGLM 1-12). The combined fraction BGLM 3-8 was purified by CC and washed using cold *n*-hexane to yield taraxerol **6** and 24-methylenelanosta-7,9(11)-diene-3β,15α-diol **7**.

Biological activities

Solvents and chemicals

Antioxidant: 1,1-diphenyl-2-picrylhydrazyl (DPPH•) and butylated hydroxytoluene (BHT) were obtained from Sigma-Aldrich (Germany). Analytical grade methanol, ethanol and dimethylsulfoxide (DMSO) were purchased from Merck (Germany). *Antimicrobial:* Nutrient agar (NA), nutrient broth (NB), sabouraud dextrose agar (SDA), sabouraud dextrose broth (SDB), streptomycin sulphate and nystatin were purchased from Oxoid (Italy). All tested microorganisms were purchased from Mutiara Scientific (Malaysia). *Anti-inflammatory:* Lipoxygenase inhibitor screening assay kit (Item No. 760700 Cayman Chemicals Co) was purchased from i-DNA Biotechnology (M) Sdn. Bhd. (Malaysia). *Acetylcholinesterase:* AChE enzyme (Type-VI-S, EC3.1.1.7), acetylthiocholine iodide, 5,50-dithio-bis(2-nitrobenzoic) acid (DTNB), and galantamine were purchased from Sigma-Aldrich (Germany). Analytical grade *n*-hexane, ethyl acetate and methanol used for extraction were purchased from Merck (Germany).

DPPH• free radical scavenging assay

The free radical scavenging activity was measured by the DPPH• method with minor modifications (16). Briefly, 0.1 mM DPPH• (1 mL) dissolved in EtOH was added to an EtOH solution (3 mL) of the tested samples and standard (BHT) at different concentrations (200, 150, 100, 50, 25 µg/mL). An equal volume of EtOH was added in the control test. The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then the absorbance at 517 nm was measured with a UV-vis spectrophotometer. The percent inhibitions (I%) of DPPH• radical were calculated as follow:

$$I\% = [\ A_{blank} - A_{sample} / A_{blank}\ ] \times 100$$

where A_{blank} is the absorbance value of the control reaction (containing all reagents except the test samples) and A_{sample} is the absorbance values of the test samples/standard. The sample concentration providing 50% inhibition (IC_{50}) was calculated by plotting inhibition percentages against concentrations of the sample. All tests were carried out in triplicate and IC_{50} values were reported as means ± SD of triplicates.

Antimicrobial activity

Microbial strains: Six microorganisms; two Gram-positive bacteria (*Bacillus subtilis* ATCC6633; *Staphylococcus aureus* ATCC29737), two Gram-negative bacteria (*Pseudomonas aeruginosa* ATCC9027 and *Escherichia coli* ATCC10536) and two fungals (*Aspergillus niger* ATCC16888 and *Candida glabrata* ATCC2001) were used. The strains were grown on NA for the bacteria and SDA for fungals. For the activity tests, NB for bacteria and SDB for fungals strains were used.

Micro-dilution method: Antimicrobial test were carried out by MIC (minimum inhibitory concentration), MBC (minimum bactericidal concentration) and MFC (minimum fungicidal concentration) by broth micro dilution method using 96-well microplates (17). The inocula of the microbial strains were prepared from 24 h broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. Samples (1 mg) were dissolved in DMSO (1 mL) to get 1000 µg/mL stock solution. A number of wells (A–H) were reserved in each plate for positive and negative controls. Sterile broth (100 µL) was added to the well from row B to H. The stock solutions of samples (100 µL) were added to the wells at row A and B. Then, the mixture of samples and sterile broth (100 µL) at row B were transferred to each well in order to obtain a twofold serial dilution of the stock samples (concentration of 1000, 500, 250, 125, 62.5, 31.3, 15.6 and 7.8 µg/mL). The inoculum (100 µL) was added to each well. The final volumes in each well were 200 µL. Streptomycin sulphate for bacteria and nystatin for fungal were used as positive controls. Plates were incubated at 37°C for 24 h. Microbial growth was indicated by the presence of turbidity and a pellet at the bottom of the well. Samples from the MIC study which did not show any growth of bacteria were removed from each well (10 mL) and then subculture on the surface of the freshly prepared nutrient agar in disposable Petri dishes (50mm×15 mm). Then, the Petri dishes were inverted and incubated for 16-20 h at 37°C. After 16-20 h, the number of surviving organisms was determined.

Lipoxygenase (LOX) inhibitory activity

LOX inhibition was determined using an enzyme immuno assay (EIA) kit (Catalog No. 760700, Cayman Chemical, USA) according to the manufacturer's instructions and

literature (8). The Cayman Chemical lipoxygenase inhibitor screening assay detects and measures the hydroperoxides produced in the lipoxygenation reaction using a purified lipoxygenase. Stock solutions of the samples were dissolved in a minimum volume of DMSO and were diluted using the supplied buffer solution (0.1 M, Tris-HCl, pH 7.4). To a 90 µL solution of 5-LOX enzyme in 0.1 M, Tris-HCl, and pH 7.4 buffer, 10 µL of various concentrations of test samples (final volume of 210 µL) were added and the lipoxygenase reaction was initiated by the addition of 10 µL (100 µM) of arachidonic acid. After maintaining the 96-well plates on a shaker for 5 min, 100 µL of chromogen was added and the plate was retained on a shaker for 5 min. The lipoxygenase activity was determined after measuring absorbance at a wavelength of 500 nm. The percentage inhibition (I%) of the samples was calculated as follows:

$$I\% = [\ A_{\text{initial activity}} - A_{\text{inhibitor}} / A_{\text{initial activity}}\ ] \times 100$$

where $A_{\text{initial activity}}$ is the absorbance of 100% initial activity wells without sample and $A_{\text{inhibitor}}$ is the absorbance of samples/reference. All tests were carried out in triplicate and expressed as means ± SD.

Acetylcholinesterase (AChE) activity

AChE inhibitory activity of the samples was measured by slightly modifying the spectrophotometric method (18-19). Electric eel AChE was used, while acetylthiocholine iodide (AChI) was employed as the substrate of the reaction. DTNB acid was used for the measurement of the AChE activity. Briefly, 140 µL of sodium phosphate buffer (pH 8.0), 20 µL of DTNB, 20 µL of test samples and 20 µL of AChE solution were added by multichannel automatic pipette in a 96-well microplate and incubated for 15 min at 25°C. The reaction was then initiated with the addition of 10 µL of AChI. Hydrolysis of AChI was monitored by the formation of the yellow 5-thio-2-nitrobenzoate anion as a result of the reaction of DTNB with thiocholines, catalyzed by enzymes at 412 nm utilizing a 96-well microplate reader (Epoch Micro-Volume Spectrophotometer, USA). Percentage of inhibition (I%) of AChE was determined by comparison of rates of reaction of samples relative to blank sample (EtOH in phosphate buffer pH = 8) using the formula:

$$I\% = [\ E - S / E\ ] \times 100;$$

where E is the activity of enzyme without test sample and S is the activity of enzyme with test sample. The experiments were done in triplicate. Galantamine was used as the positive controls.

Statistical analysis

Data obtained from the biological activities are expressed as mean ±SD of triplicates. The statistical analyses were carried out by employing one-way ANOVA. A statistical package (SPSS version 11.0) was used for the data analysis.

RESULTS AND DISCUSSION

Compound **1** was obtained as a colourless liquid. The EIMS spectrum revealed the molecular ion peak at *m/z* 266 and the molecular formula was deduced as $C_{16}H_{26}O_3$, with four degree of unsaturation, indicative of one cyclic and three double bonds. The IR spectrum showed absorption bands for a hydroxyl group at 3418 cm^{-1}, and an α,β-unsaturated γ-lactone moiety at 1774 and 1675 cm^{-1}. The UV spectrum showed an absorption at 245 nm indicating the presence of β-hydroxy-γ-methylene-α,β-unsaturated γ-lactone (20). The ^{1}H NMR and COSY spectra showed the presence of β-hydroxy-γ-methylene-α,β-unsaturated γ-lactone skeleton with the *Z* geometry of the trisubstituted double bond at δ 6.68 (1H, ddd, *J* = 2.4, 2.0, 2.0 Hz) corresponding to proton H-1′. The presence of a broad singlet δ 1.27 integrating for fourteen protons was assigned to protons in an aliphatic chain, H-4′–10′. The methyl group for H-11′ was observed as a triplet at δ 0.90 (*J* = 6.4 Hz). The exocyclic olefinic protons appeared as doublet of doublets at δ 4.69 and 4.90 (each *J* = 2.8, 1.6 Hz, H-6a/b), and one singlet signal of hydroxymethine proton was observed at δ 5.13 (H-4). The position of these groups was established from the HMBC spectrum. The exocyclic olefinic signals at δ 4.69 and 4.90 were correlated with a quaternary carbon at δ 157.5 (C-5) and a methine carbon at δ 68.9 (C-4). The former carbon also correlated with the signal at δ 5.13 (H-4). The HMQC spectrum revealed direct connectivity between protons H-6a (δ 4.69) and H-6b (δ 4.90) with C-6 (δ 90.2), H-4 (δ 5.13) with C-4 (δ 68.9), and H-1′ (δ 6.68) with C-1′ (δ 151.3). The geometry of the alkylidene side chain was *cis* to the carbonyl group, confirmed in the NOESY spectrum which showed cross-peaks between H-4 and H-1′. The configuration at C-4 was determined as 4*R* on the basis of their specific rotation, +34.2 (*c* 0.15, $CHCl_3$) and the configuration at C-4 for 3-alkylidene-4-hydroxyl-5-methylenebutanolide derivatives (21-22). The ^{13}C NMR spectrum showed sixteen carbon signals. The DEPT spectra characterized these carbons as one methyl, two methines, ten methylenes, and three quaternary carbons. Thus, the structure of compound **1** was elucidated as (4*R*,3*Z*)-4-hydroxy-5-methylene-3-undecylidenedihydrofuran-2-one or known as subamolide D (10).

Compound **2** was obtained as a colourless liquid. The EIMS spectrum revealed the molecular formula $C_{16}H_{26}O_3$, corresponding to the molecular ion peak at *m/z* 266. In the IR spectrum, the α,β-unsaturated γ-lactone moiety were observed at 1777 and 1670 cm^{-1}, while the hydroxyl group was observe at 3472 cm^{-1}. The ^{1}H NMR spectrum of compound **2** was similar to that of **1** indicating the presence of β-hydroxy-γ-methylene-α,β-unsaturated γ-lactone skeleton. The trisubstituted double bond observed at δ 7.07 (1H, ddd, *J* = 2.4, 2.0, 2.0 Hz) assigned for H-1′. The geometry of the alkylidene side chain was *trans* to the carbonyl group on the basis of the chemical shifts of H-1′ (δ 7.07) and H-2′ (δ 2.43) in the ^{1}H NMR spectrum. The downfield shift of H-1′ (δ 7.07) compared with that of H-1′ (δ 6.68) in compound **1** can be ascribed to the effect of the carbonyl group of a lactone ring. The position of these groups was established from the HMBC spectrum. Its *trans* geometry was also confirmed in the NOESY spectrum, which showed cross-peaks between H-4 and H-2′. The specific rotation of compound **2** was +20.8 (*c* 0.15, $CHCl_3$), while the stereochemistry of C-4 of compound **2** was similar to that of **1** (21-22). The ^{13}C NMR spectrum furnished sixteen signals attributed to sixteen carbons. The DEPT spectra characterized these signals as one methyl carbon at δ 14.1 (C-11′), two methine carbons at δ 66.4 (C-4), 150.3 (C-1′), three quaternary carbons at δ 28.3 (C-3), 127.2 (C-3), 157.6 (C-5), and ten methylene carbons at δ 22.6 (C-10′), 29.3–29.6 (C-4′–8′), 29.7 (C-2′), 31.9 (C-9′), 91.4 (C-6), 166.8 (C-2). Thus, the structure of compound **2** was elucidated as (4*R*,3*E*)-4-hydroxy-5-methylene-3-undecylidenedihydrofuran-2-one or known as subamolide E (10). Other compounds, β-sitosterol **3** (11), β-sitostenone **4** (12), lupeol **5** (13), taraxerol **6** (14), and 24-methylenelanosta-7,9(11)-diene-3β,15α-diol **7** (15) were identified by comparison of their spectroscopic data with published data.

Biological activities of butanolides, such as anti-inflammatory (23), cytotoxicity (24), and anti-complement (25) have been reported to date. The isolate compounds isolated in this study, were tested for their antioxidant, antimicrobial, acetylcholinesterase and anti-inflammatory activities. The results are shown in Table 1. The antioxidant activity was evaluated by using the DPPH• free-radical-scavenging with a series of different concentrations. Compounds **1** and **2** showed weak antioxidant activity with IC_{50} values of 315.2 and 356.4 μM, respectively. The antimicrobial activity of the compounds exhibited from moderate to weak activities with MIC values ranging from 250–1000 μg/mL. The results are shown in Table 2. All compounds have shown weak AChE inhibitory activity using the Ellman's colorimetric method. In the LOX assay, compound **1** and **2** furnished strong activity with IC_{50} the values of 5.1 and 5.5 μM, respectively. The IC_{50} value for quercetin, used as a positive control, was 3.5 μM.

The chemistry of the Lauraceae family is recognized by the presence of butanolides, which isolated previously from the genus of *Litsea* (26), *Lindera* (23), *Machilus* (24), *Cinnamomum* (27), and *Alseodaphne* (28). Meanwhile, subamolides D-E have been isolated previously from the leaves of *C. subavenium* (10). Both compounds **1**-**2** are similar lactonic type compounds, subamolides A-C which had been isolated previously from the stem parts of *C. subavenium* (27). Subamolide A, an isomer of subamolide B, has been reported to induce apoptosis in human colon adenocarcinoma cell line SW480 and human urothelial carcinoma cell line NTUB1 in addition to acting as an inhibitor of human tyrosinase (27, 29-30). Furthermore, an *in vitro* antimelanoma activity has been assigned to subamolide E (10,31). As to the bioactivity of subamolide B, it has an ability to induce apoptosis in SW480 cells (27).

Table 1. Antioxidant, acetylcholinesterase, and lipoxygenase inhibitory activities of the isolated compounds from *B. glabra*

Compounds	**DPPH• IC_{50}** (μM)	**LOX IC_{50} (μM)**	**AChE IC_{50} (μM)**
(**1**)	315.2	5.1	NA
(**2**)	356.4	5.5	NA
(**5**)	NA	35.0	91.3
(**6**)	NA	54.1	79.5
(**7**)	NA	30.5	NA
BHT	84.0	-	-
Quercetin	-	3.5	-
Galantamine	-	-	40.7

Data represent as mean ± SD of three independent experiments; NA – not active

Table 2. Antimicrobial activity of the isolated compounds from *B. glabra*

Compounds/ Microbes		*Bacillus subtilis*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Escherichia coli*	*Aspergillus niger*	*Candida glabrata*
(**1**)	**MIC**	250	250	500	1000	250	1000
	MBC	500	500	1000	>1000	500	>1000
(**2**)	**MIC**	250	250	500	1000	250	1000
	MBC	500	500	1000	>1000	500	>1000
(**5**)	**MIC**	250	250	250	500	250	250
	MBC	250	500	250	500	500	250
(**6**)	**MIC**	500	500	1000	1000	500	500
	MBC	1000	1000	>1000	>1000	1000	1000
(**7**)	**MIC**	500	500	1000	1000	500	1000
	MBC	500	500	>1000	>1000	1000	1000
SS	**MIC**	7.8	7.8	7.8	7.8	ND	ND
	MBC	7.8	7.8	7.8	7.8	ND	ND
NYS	**MIC**	ND	ND	ND	ND	7.8	7.8
	MBC	ND	ND	ND	ND	7.8	7.8

Results of MIC/MBC/MFC in µg/mL; SS - Streptomycin sulphate; NYS – Nystatin; ND – not determined

CONCLUSION

To the knowledge of the authors, this is the first report on butanolides from genus *Beilschmiedia*. This study revealed that the butanolides showed promising lipoxygenase inhibitory activity but poor DPPH and AChE activities. The isolated compounds should be further evaluated to develop safe agents to be introduced in modern therapy. Further studies should be made to reveal the mode of action of butanolides which might be helpful in understanding possible roles in human physiology.

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***Beilschmiedia glabra* yapraklarından izole edilen kimyasal bileşenlerin biyolojik etkinleri**

ÖZ

Beilschmiedia glabra yapraklarından, butanolit türevi iki bileşik ; subamolit D (**1**) ve subamolit E (**2**) ile birlikte β-sitosterol (**3**), β-sitostenon (**4**), lupeol (**5**), tarakserol (**6**), and 24-metilenlanosta-7,9(11)-dien-3β,15α-diol (**7**) de izole edilmiştir. İzole edilen bileşiklerin yapıları spektroskopik yöntemlerle aydınlatılmış ve elde edilen veriler literatür verileri ile karşılaştırılmıştır. Bileşikler, DPPH• radikal tutucu, antimikrobiyel, antikolinesteraz ve lipooksijenaz inhibitor etkileri açısından değerlendirilmişlerdir. Bileşik **1** ve **2**, sırasıyla 5.1 ve 5.5 µM IC_{50} değerleri ile en yüksek lipooksijenaz inhibitörü etkinliği göstermişlerdir. Tüm bileşikler; zayıf DPPH• radikal tutucu, antimikrobiyel, asetilkolinesteraz inhibitörü etkinlik göstermişlerdir.

Keywords: Lauraceae, *Beilschmiedia*, DPPH•, antimikrobiyel, asetilkolinesteraz, lipooksijenaz

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