ISOLATION OF SECONDARY METABOLITES FROM MEDICINAL PLANTS AND INVASIVE ALIEN SPECIES AND THEIR BIOLOGICAL ACTIVITIES

ANTONI PARDEDE

February 2018

ISOLATION OF SECONDARY METABOLITES FROM MEDICINAL PLANTS AND INVASIVE ALIEN SPECIES AND THEIR BIOLOGICAL ACTIVITIES

MATERIALS ENGINEERING DIVISION
GRADUATE SCHOOL OF ENGINEERING
GIFU UNIVERSITY
JAPAN

ANTONI PARDEDE

December 2017

Preface

The studies presented in this thesis have been carried out under the guidance of Professor Mamoru Koketsu at Materials Engineering Division, Graduate School of Engineering, Gifu University, during 2015-2018.

The studies are concerned with isolation of secondary metabolites from medicinal plants and invasive alien species and their biological activities.

February 2018

Antoni Pardede

Summary

Plants are the tremendous source for the discovery of secondary metabolites with biological activity developments. In this study, the isolation of secondary metabolites from medicinal plants and invasive alien species and their biological activities were investigated.

This thesis consists of 4 chapters. The first chapter describes antioxidant and antileukemic activity of chemical constituents from bark of *Mangifera casturi*. The second chapter describes flavonoid rutinosides from *Cinnamomum parthenoxylon* leaves and their hepatoprotective activity. The third chapter is isolation of secondary metabolites from *Stenochlaena palustris* stems and structure activity relationship (SAR) of 20-hydroxyecdysone derivatives on antitermite activity. Finally, the fourth chapter describes chemical constituents of *Coreopsis lanceolata* stems and their antitermite activity against *Coptotermes curvignathus*.

In chapter 1, the antioxidant and antileukemic activity of chemical components from bark of *Mangifera casturi* was investigated. Research findings have shown that several extracts of *Mangifera casturi* have potent bioactivity. The methanol extract of *Mangifera casturi* bark was partitioned successively to yield *n*-hexane (*n*-Hex) fraction (6.7%), ethyl acetate (EtOAc) fraction (24.1%) and *n*-butanol (*n*-BuOH) fraction (28.1%). Five compounds were isolated from EtOAc fraction and one compound was isolated from *n*-hexane fraction. These compounds were identified as methyl gallate (**1**), taxifolin (**2**), pyrocatechuic acid (**3**), gallic acid (**4**), glucogallin (**5**), and β-sitosterol (**6**), respectively; they were

confirmed by spectroscopic analysis and ultra-performance liquid chromatography electrospray ionization time-of-flight mass spectrometry (UPLC-ESITOFMS). All compounds were isolated from bark of *Mangifera casturi* for the first time. The EtOAc fraction as well as the isolated gallic acid (**4**) showed potent antioxidative and antileukemic activity against Human Leukemia HL-60 cells.

In chapter 2, flavonoid rutinosides were isolated from *Cinnamomum parthenoxylon* leaves and their hepatoprotective and antioxidant activity were evaluated. The EtOAc fraction of *C. parthenoxylon* leaves showed potent hepatoprotective activity on *tert*-butyl hydroperoxide (*t*-BHP) induced cytotoxicity in human hepatoma (HepG2) cells and also higher antioxidant activity. UPLC-ESITOFMS analysis revealed that flavonoid rutinosides; rutin (**6**), nicotiflorin (**9**), and isorhoifolin (**2**) are major constituents in the EtOAc fraction. The catechol group on B ring in the structure of rutin holds potential for hepatoprotective and antioxidant activity.

In chapter 3, the effects of fractions of *Stenochlaena palustris* stems and isolated constituents on termite feeding behavior and mortality were studied. Treatment of the ethyl acetate (EtOAc) fraction on paper discs greatly led to the death of *Coptotermes curvignathus* within 6 days. The phytochemical investigation of *S. palustris* stems led to the isolation of major constituents and 20-hydroxyecdysone exerted the highest termicidal activity, followed by stenopalustroside A and ajugasterone C. Moreover, the structure activity relationships of synthetic derivatives from 20-hydroxyecdysone and ajugasterone C suggested that a 2,3-diol of them has considerable effect on their antitermite properties against *C. curvignathus*.

In chapter 4, the chemical constituents were isolated from *Coreopsis lanceolata* stems and their antitermite activity was evaluated. *Coreopsis lanceolata* is an *Asteraceous* plant having high fertility and resistance to pathogenic organisms. The working purpose is the discovery of termite resistant constituents from *C. lanceolata* stems. Our phytochemical research led to the isolation of major components. Their antitermite effects were evaluated with the no-choice test against *Coptotermes curvignathus*. Of the isolates, 5-phenyl-2-(1-propynyl)-thiophene and 1-phenylhepta-1,3,5-triyne showed strong potent antitermite activity. Our findings suggested that *C. lanceolata* appears to be an antitermite material.

In conclusions, the isolation of secondary metabolites from medicinal plants and invasive alien species and their biological activities was investigated. The gallic acid compound isolated from bark of *M. casturi* showed potent antioxidant activity and antileukemic activity against human leukemia HL-60 cells. Further, rutin compound isolated from *C. parthenoxylon* leaves showed high hepatoprotective and antioxidant activity among the investigated flavonoid rutinosides. More attention must be given because *S. palustris* stems possessed the excellent potential as the natural resource of ecdysteroids. The application of phenyl-2-(1-propynyl)-thiophene, 1-phenylhepta-1,3,5-triyne and 20-hydroxyecdysone compound isolated from *C. lanceolata* stems and *S. palustris* stems, respectively, to be used as antitermite materials.

Table of Content

Abbreviations

δ	Chemical shift (ppm)
2D NMR	Two dimention NMR
Ac_2O	Acetic anhydrous
AcOH	Acetic acid
CC	Column chromatography
CH_3CN	Acetonitrile
CH_2Cl_2	Dicloromethane
$CHCl_3$	Chloroform
COSY	Correlation spectroscopy
d	doublet
DEPT	Distortionless enhancement by polarization transfer
DMSO	Dimethyl sulfoxide
EtOAc	Ethyl acetate
Fr	Fraction
HMBC	Heternuclear multiple bond connectivity
HMQC	Heteronuclear multiple quantum coherence
HREIMS	High resolution electron ionization mass spectrometry
HRESITOFMS	High resolution electrospray ionization time-of-flight mass spectrometry
Hz	Hertz
IR	Infrared Spectroscopy
J	Coupling constant (Hz)

m/z	Mass to charge ratio
MeOH	Methanol
MS	Mass spectroscopy
Na_2SO_4	Sodium sulfate
$NaHCO_3$	Sodium hydrogen carbonate
n-BuOH	Normal butanol
n-Hex	normal hexane
NMR	Nuclear magnetic resonance
PTLC	Preparative thin layer chromatography
q	Quartet
r.t	Room temperature
RH	Relative humidity
s	Singlet
SiO_2	Silica gel
t	triplet
TLC	Thin layer chromatography
UPLC-ESITOFMS	Ultra performance liquid chromatography electrospray ionization time-of-flight mass spectrometry
$ZnCl_2$	Zinc chloride

General Introduction

Plants are surrounded by an enormous number of potential enemies in their natural habitats. Nearly all ecosystems contain a wide variety of bacteria, fungi, mites, insects, nematodes and other herbivorous animals. By their nature, plants cannot avoid these pathogens and herbivores simply by moving away; they must protect themselves in other ways. Plants produce large numbers of organic compounds that appear to have functioned in defend plants against a variety of herbivores and pathogenic microbes. These organic compounds are known as secondary metabolites (Mazid et al., 2011).

A simple classification of secondary metabolites includes tree main groups: terpenes (such as volatiles compound, carotenoids and sterols), phenolics (such as phenolic acids, coumarins, lignans, stilbenes, flavonoids, tannins and lignins) and nitrogen containing compounds such as alkaloids. A number of separation techniques with various stationary and mobile phases of column chromatography, spray reagents and spectroscopic analysis have been described as having the ability to separate and identify secondary metabolites (Agustini-Costa et al., 2012). The secondary metabolites from plants possesses a wide spectrum of biological properties, such as antileukemia, antioxidant, antibacterial, hepatoprotective, antitermite and insecticidal activities (George et al., 2011; Kakumu et al., 2014; Ahmad et al., 2015).

Republic of Indonesia since its independence on August 17, 1945 has the motto “Unity in Diversity” which is expressing the condition of Indonesia.

Diversity in the motto refers to ethnics, religions, languages, cultures and natural resources including plants, wildlife, mineral etc (Damayanti et al., 2011).

The Indonesian plants were utilized as medicine that has been in traditional recipes bequeathed by the ancestors of Indonesian people. Indonesian plants are a remarkable opportunity for the development of secondary metabolites for biological activities. More researches are necessary to reveal the secondary metabolites in the Indonesian plants and biological assay system such as anticancer, antidiabetic, antibacterial, antioxidant, antitermite and insecticidal properties. The secondary metabolites such as terpens, phenolics and alkaloids reported had been responsible for the biological activities (Ninomiya et al., 2013; Adfa et al., 2016; Sholikhah, 2016; Dos Santos et al., 2017; Joshi et al., 2017).

Recently, isolation of secondary metabolites of terpens, phenolics and alkaloids has been reported from various parts of the Indonesian plants. Three clerodane diterpenes, kolavenic acid, polyalthialdoic acid and 16α-hydroxy-cleroda-3,13(14)*Z*-dien-15,16-olide isolated from *Polyalthia longifolia* leaves induce apoptotic death in the human leukemia HL-60 cells. The resuts revealed that the *P. longifolia* is important as a chemopreventive medicinal plant (Sari et al., 2013). Further, Kakumu et al. (2014) have described a phytochemical study on *Toona sinensis* and isolated several polyphenolic constituents. The gallic acid and loropetalin D isolated from *Toona sinensis* showed the strong activity against human leukemia HL-60 cells. Two alkaloid compounds (*N-trans*-feruloyltyramine and salicifoline chloride) successively isolated from branches of *Enicosanthum membranifolium* (Efdi et al., 2007). The coumarins compounds also isolated from Indonesian plants (*Protium javanicum* Burm. f.) reported by Adfa et al. (2010). As

the results, scopoletin exhibited the strongest termiticidal activity against *Coptotermes formosanus* Shiraki. The structures of chemical constituents isolated from Indonesian plants (*Polyalthia longifolia*, *Toona sinensis, Enicosanthum membranifolium* and *Protium javanicum* Burm. f.) were shown in **Figure 1**.

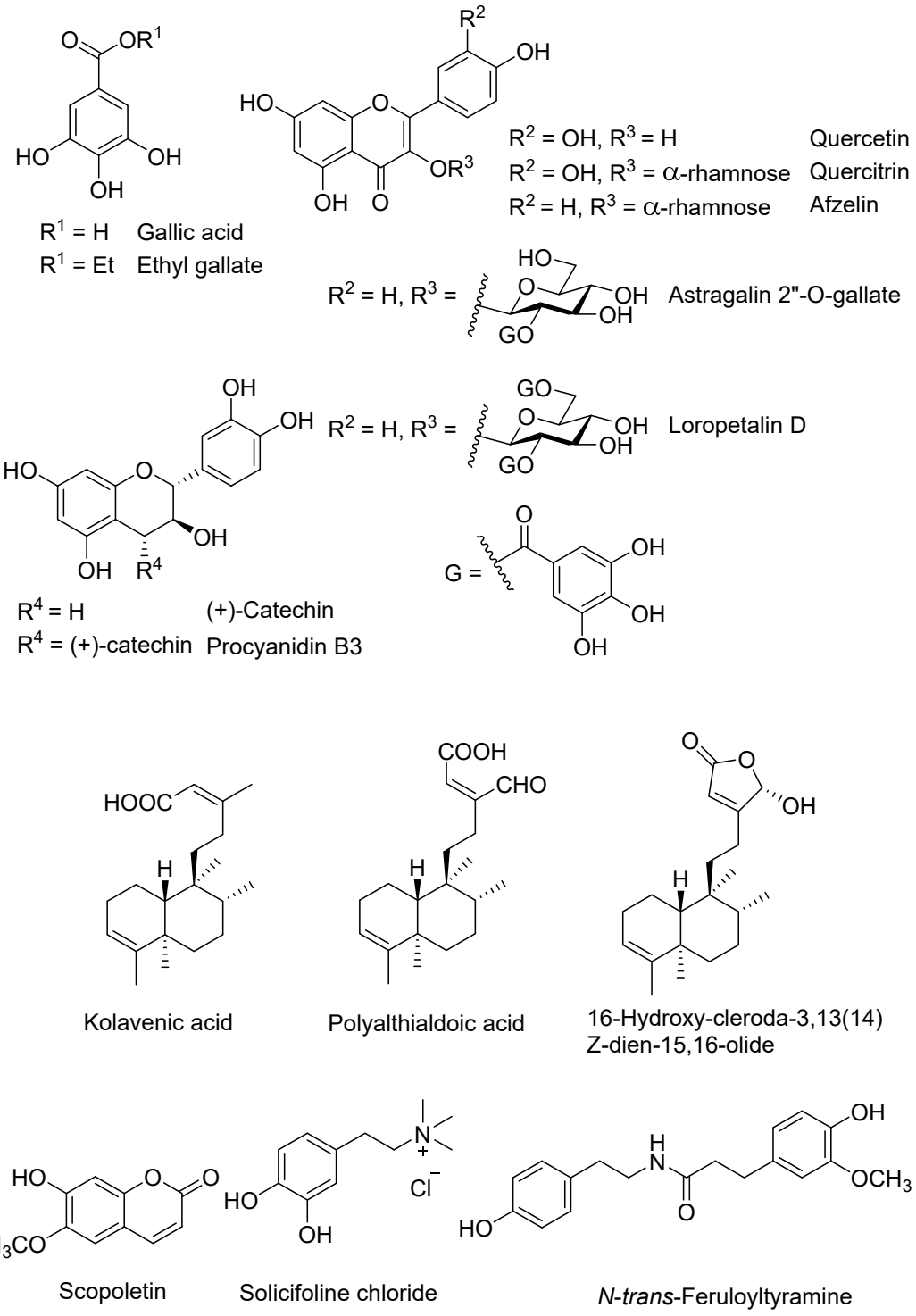


Figure 1. The structures of the chemical constituents isolated from Indonesian plants (*Polyalthia longifolia*, *Toona sinensis, Enicosanthum membranifolium* and *Protium javanicum* Burm. f.).

Due to the diversity of secondary metabolites and biological activities of the Indonesian plants, we are interested in isolation and bioactivity of the secondary metabolites of Indonesian plants. For this purpose, we have collected three Indonesian plants (*Mangifera casturi* bark*, Cinnamomum parthenoxylon* leaves and *Stenochlaena palustris* stems). In addition, *Coreopsis lanceolata* stems was collected around Gifu city, Japan. *Coreopsis lanceolata* is native in North America and now widely distributed in Asia and Oceania regions. Previously, because this plant produces beautiful flowers in spring, it was planted for decoration on the roadside and bankside in Japan. However, *C. lanceolata* has high fertility; it is recognized as the invasive alien species today. The *C. lanceolata* stems had been selected in this research base on our previously phytochemical study. We identified rare flavonoid including a flavonone, chalcones and aurones were major constituents of the *Coreopsis lanceolata* flowers and they displayed potent antileukemic activity (Pardede et al., 2016).

Mangifera casturi (Anacardiaceae) is an endemic plant from South Kalimantan Indonesia. It is called as mangga kasturi or mangga Borneo in Indonesia, which has several applications in traditional medicine (Suhartono et al., 2012).

Cinnamomum parthenoxylon tree belongs to the *Lauraceae* family. It is called kayu gadis and has been used by local people as spices in foods, fragrances, fumigants, and traditional medicines in Indonesia (Wang et al, 2013; Kawatra and Rajagopalan, 2015). *Cinnamomum parthenoxylon* is a large, evergreen tree species, grows as high 10 - 30 meters. The bark is silvery grey, smooth and

slightly fragrant. The leaves occur singly, upper layer is glabrous, whereas the lower layer is brilliantly green and slightly fragrant (Sein and Mitlöhner, 2011).

Stenochlaena palustris is an edible fern which is commonly referred to as kelakai in South Kalimantan Indonesia, belongs to *Blechnaceae* family. The reddish, young sterile fronds of the fern are harvested from the wild, consumed as vegetable and commonly sold on local market (Chai et al., 2012).

Coreopsis lanceolata is a kind of perennial plants that belongs to the *Asteraceae* family. This plant has high fertility, produces beautiful flowers and plants for decoration on the homested (Trader et al., 2006).

The purposes of this research are to isolate the secondary metabolites from *Mangifera casturi* bark*, Cinnamomum parthenoxylon* leaves, *Stenochlaena palustris* stems and *Coreopsis lanceolata* stems and investigated their biological activity such as antileukemia, antioxidant, hepatoprotective and antitermite activity. In addition, in the chapter 3, synthesis of 20-hydroxyecdysone derivatives and their structure activity relationship (SAR) on antitermite activity also investigated in this study.

References

Adfa, M., Rahmad, R., Ninomiya, M., Yudha, S. S., Tanaka, K., Koketsu, M. 2016. Antileukemic activity of lignans and phenylpropanoids of *Cinnamomum parthenoxylon*. Bioorg. *Med. Chem. Lett. 26*:761-764.

Adfa, M., Yoshimura, T., Komura, K., Koketsu, M. 2010. Antitermite activities of coumarin derivatives and scopoletin from *Protium javanicum Burm. f. J. Chem. Ecol. 36*:720-726.

Agustini-costa, T. D. S., Vieira, R. F., Bizzo, H. R., Silviera, D., Gimenes, M. A., 2012. Secondary metabolites. *Chromatograp and its applications.* p. 131.

Ahmad, S., Sukari, M. A., Ismail, N. 2015. Phytochemicals from *Mangifera pajang* Kosterm and their biological activities. *Compl. Alter. Med. 15*:83.

Chai, T. T., Panirchellvum, E., Ong, H. C., Wong, F. C. 2012. Phenolic contents and antioxidant properties of *Stenochlaena palustris*, an edible medicinal fern. *Biochem. 53*:439-446.

Damayanti, E. K., Hikmat, A., Zuhud E. A. M. 2011. Indonesia tropical medicinal plants diversity: problems and challenges in identification. International workshop "linking biodiversity and computer vision technology to enhance sustainable utilization of Indonesian tropical medicinal plants. p. 1-17.

Dos Santos, AR., Pires, C., Marques, F. A., Lobão, A. Q., Maia, B. H. L. N. S. 2017. Isoquinoline alkaloids isolated from three Guatteria species. *Biochem. Syst. Ecol. 73*:1-2.

Efdi, M., Itoh, T., Akao, Y., Nozawa, Y., Koketsu, M., Ishihara H. 2007. The isolation of secondary metabolites and in vitro potent anti-cancer activity of clerodermic acid from *Enicosanthum membranifolium*. *Bioorg. Med. Chem. 15*:3667-3671.

George, S., Benny, P. J., Sunny, K., Cincy, G. 2011. Antibiotic activity of 2, 3-dihydroxybenzoic acid isolated from *Flacourtia inermis* fruit against multidrug resistant bacteria. *Asian J. Pharm. Clin. Res. 4*:126-130.

Joshi, P., Vishwakarma, RA., Bharate., SB. 2017. Natural alkaloids as P-gp inhibitors for multidrug resistance reversal in cancer. Eur. *J. Med. Chem. 138*: 273-292.

Kakumu, A., Ninomiya, M., Efdi, M., Adfa, M., Hayashi, M., Tanaka, K., Koketsu, M. 2014. Phytochemical analysis and antileukemic activity of poliphenolic constituents of *Toona sinensis*. *Bioorg. Med. Chem. Lett. 24*:4286-4290.

Kawatra, P., Rajagopalan, R. 2015. Cinnamon: Mystic powers of a minute ingredient. *Pharmacognosy Res. 7*:1-6.

Mazid, A., Khan, T. A., Muhammad, F. 2011. Role of secondary metabolites in defense mechanisme of plants. *Biol. Med. 3*: 232-249.

Ninomiya, M., Nishid, K., Watanabe, K., Koketsu, M. 2013. Structure-activity relationship studies of 5,7-dihydroxyflavones as naturally occurring inhibitors of cell proliferation in human leukemia HL-60 cells. *J. Nat. Med. 67*:460-467.

Pardede, A., Mashita, K., Ninomiya, M., Tanaka, K., Koketsu, M. 2016. Flavonoid profile and antileukemic activity of *Coreopsis lanceolata* flowers. *Bioorg. Med. Chem. Lett. 26*:2784-2787.

Sari, D. P., Ninomiya, M., Efdi, M., Santoni, A., Ibrahim, S., Tanaka, K., Koketsu, M. 2013. Clerodane diterpenes isolated from *Polyalthia longifolia* induce apoptosis in human leukemia HL-60 cells. *J. Oleo Sci. 62*:843-848.

Sein, C. C., Mitlöhner, R. 2011. *Cinnamomum parthenoxylon* (Jack) Meisn. ecology and silviculture in Vietnam. Center for International Forestry Research p. 1-2.

Sholikhah, E. N. 2016. Indonesian medicinal plants as sources of secondary metabolites for pharmaceutical industry. *J. Med. Sci. 48*:226-239.

Suhartono, E., Viani, E., Rahmadhan, M. A. 2012. Total flavanoid and antioxidant activity of some selected medicinal plants in South Kalimantan of Indonesia. *Asia Pacific Chem. Biol. Environ. Eng. 4*:235-239.

Trader, B. W., Gruszewski, H. A., Scoggins, H. L., Veilleux, R. E. 2006. Somaclonal variation of Coreopsis regenerated from leaf explants. *Hortscience 41*:749-752.

Wang, Y. H., Avula, B., Nanayakkara N. P., Zhao, J., Khan, I. A. 2013. *Cassia Cinnamon* as a source of coumarin in Cinnamon-Flavored food and food supplements in the United States. *J. Agric. Food Chem. 61*:4470-4476.

Chapter 1

Antioxidant and antileukemic activity of chemical components from bark of *Mangifera casturi*

1.1. Introduction

Mangifera casturi (*Anacardiaceae*) is an endemic plant from South Kalimantan, Indonesia. It is called as mangga kasturi or mangga Borneo in Indonesia, which has several applications in traditional medicine (Suhartono et al., 2012). The picture of *Mangifera casturi* part is show in **Figure 2.**

a b c d

Figure 2. Part of *Mangifera casturi*, fruits (a), tree (b), leave (c) and grounded bark (d).

Chemical components from plants such as phenols, flavonoids, and terpenoids have shown a wide array of biological activities such as antioxidant, anticancer, antimicrobial, antiinflammatory and antidiabetic properties (George et al., 2011; Kakumu et al., 2014; Ahmad et al., 2015; Pardede et al., 2016).

The extract and chemical components from the bark and fruit of *Mangifera indica* showed antioxidant and aggressive activity against breast cancer cells (Rodeiro et al., 2006; Rivera et al., 2011; Afifa et al., 2014; Meneses et al., 2015). The crude extracts and isolated compounds from *Mangifera pajang* could be confirmed as anticancer, antimicrobial, and free radical scavenging agents (Ahmad et al., 2015). There are only a few phytochemical studies on *Mangifera casturi.* The methanol extract and lupeol (terpenoids compound) of *Mangifera casturi* fruit are reported as significant antioxidative, antiinflammatory and immunomodulatory activities (Suhartono et al., 2012; Sutomo et al., 2013; Fakhrudin et al., 2013). The chemical structure of lupeol is show in **Figure 3**.

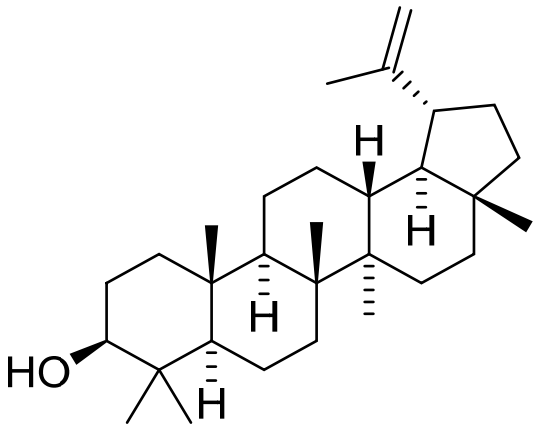


Figure 3. The chemical structure of lupeol.

However, there has been no investigation on *Mangifera casturi* bark. In this study, the chemical components were isolated from *Mangifera casturi*, and confirmed their antioxidative and antileukemic activity.

1.2. Materials and Methods

1.2.1. General experimental procedures

^{1}H and ^{13}C NMR spectra were recorded on a JEOL ECA 600/400 spectrometer with tetramethylsilane (TMS) as an internal standard. MS spectra were obtained using a JEOL JMS-700/GI spectrometer and the waters UPLCMS system (Aquity UPLC XevoQTof). Column chromatography (CC) was performed on a neutral silica gel (Silica Gel 60 N, spherical, neutral, 40-50 μm) (KANTO Chemical Co., Inc.). Preparative thin-layer chromatography (PTLC) was performed on silica gel 60 F_{254} (1 mm layer thickness Merck).

1.2.2. Plant material

The bark of *Mangifera casturi* was collected from Pengaron, South Kalimantan, Indonesia.

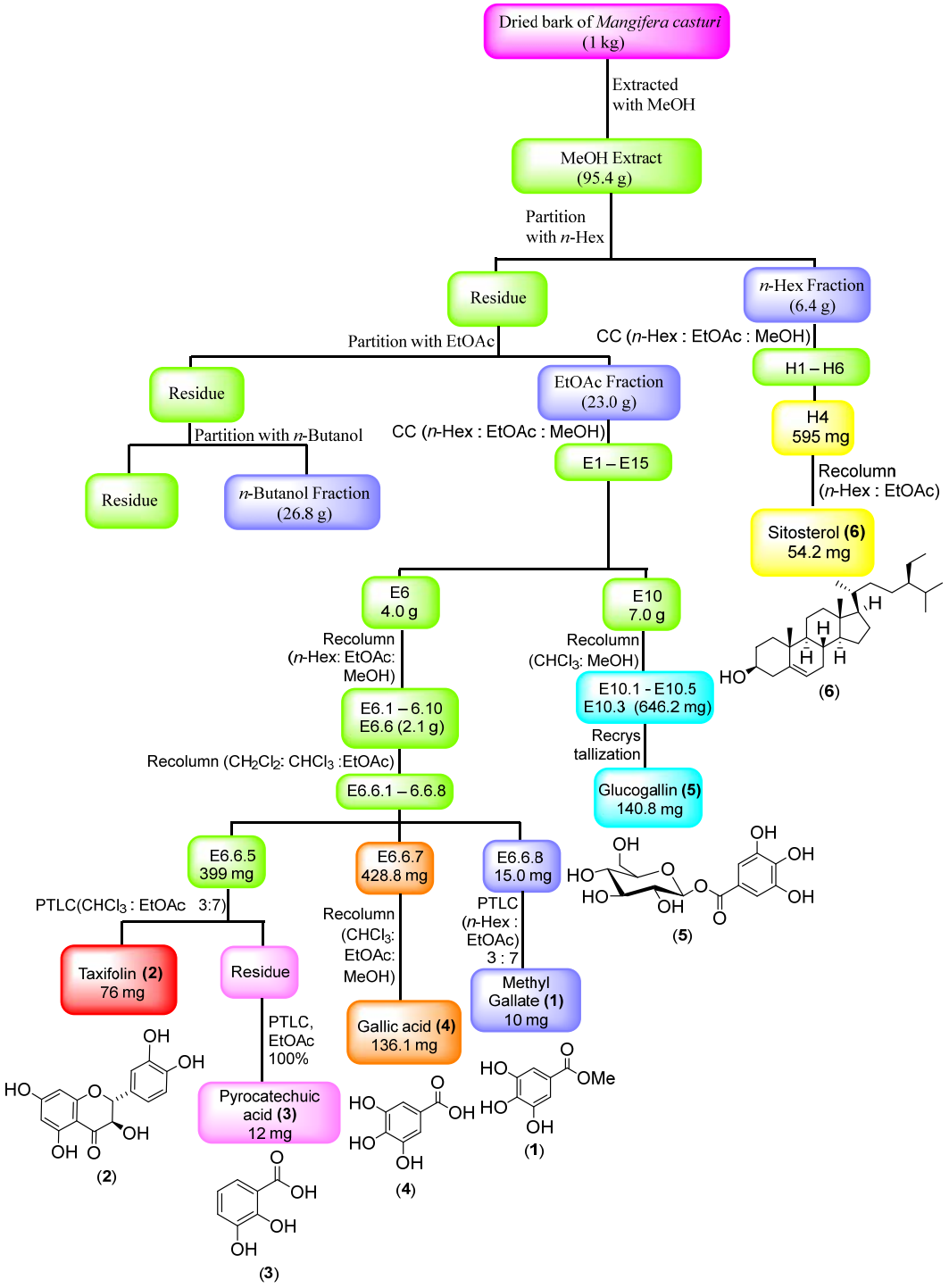
1.2.3. Extraction and isolation of chemical components from bark of Mangifera casturi

Dried bark of *M. casturi* (1 kg) was macerated with methanol at room temperature (3 x 2.5 L). The extract was filtered and evaporated *in vacuo* to yield methanol extract (95.4 g). The methanol extract was partitioned successively with

n-hexane, EtOAc, and *n*-BuOH to yield *n*-Hex fraction (6.4 g), EtOAc fraction (23.0 g), and *n*-BuOH fraction (26.8 g).

A portion of EtOAc fraction (23.0 g) was separated by silica gel column chromatography (CC) eluted with, *n*-hexane - EtOAc, EtOAc - MeOH, which yielded 15 fractions (E_1 - E_{15}). Fraction E_6 (4.0 g) was subjected to CC on silica gel (*n*-hexane - EtOAc) (EtOAc - MeOH) in a stepwise manner (10/0 to 0/10) to give 10 subfractions ($E_{6.1}$ - $E_{6.10}$). Subfraction $E_{6.6}$ (2.1 g) was subjected to CC on silica gel eluted with CH_2Cl_2 - $CHCl_3$, then $CHCl_3$ - EtOAc to obtain 8 subfractions ($E_{6.6.1}$ - $E_{6.6.8}$). Subfraction $E_{6.6.8}$ (15.0 mg) was further separated by PTLC (*n*-hexane - EtOAc 3:7) to obtain compound **1** (10 mg). Subfraction $E_{6.6.5}$ (399 mg) was separated by PTLC ($CHCl_3$: EtOAc = 3:7) to obtain compound **2** (76 mg), subfraction of $E_{6.6.5}$ was further separated by PTLC (EtOAc 100%) to obtain compound **3** (12 mg). Subfraction $E_{6.6.7}$ (428.8 mg) was subjected to CC on silica gel eluted with $CHCl_3$ – EtOAc, then EtOAc - MeOH in a stepwise manner (10/0 to 0/10) to obtain compound **4** (136.1 mg). Fraction E_{10} was subjected to CC on silica gel ($CHCl_3$ - MeOH) also in a stepwise manner of increasing polarity (10/0 to 0/10), yielding 5 fractions ($E_{10.1}$ - $E_{10.5}$). Compound **5** (140.8 mg) was isolated from fraction $E_{10.3}$ (646.2 mg) by re-crystallization.

A portion of *n*-Hex fraction (6.4 g) was subjected to CC on silica gel (*n*-hexane - EtOAc) (EtOAc - MeOH) in a stepwise manner (10/0 to 0/10) to give 6 fractions (H_1 - H_6). Fraction H_4 (595 mg) was subjected to CC on silica gel (*n*-hexane - EtOAc) to obtain compound **6** (54.2 mg) (**Scheme 1**).



Scheme 1. Isolation scheme of *Mangifera casturi* bark extract.

1.2.4. Spectral data of isolated compounds

Methyl gallate ***(1)***

White powder, HRESITOFMS *m/z* 183.0274 $[M-H]^-$ (calcd. for $C_8H_7O_5$, 183.0293). ^1H NMR (400 MHz, CD_3OD): δ 6.99 (2H, s, H-2 and H-6), 3.77 (3H, s, OCH_3); ^{13}C NMR (100 MHz, CD_3OD): δ 167.7, 145.2, 138.4, 120.1, 108.7, 50.9.

Taxifolin ***(2)***

Yellow crystals, HRESITOFMS *m/z* 303.0479 $[M-H]^-$ (calcd. for $C_{15}H_{11}O_7$, 303.0505). ^1H NMR (600 MHz, CD_3OD): δ 6.97 (1H, d, *J* = 1.9 Hz, H-2'), 6.84 (1H, dd, *J* = 8.2 and 2.0 Hz, H-6'), 6.80 (1H, d, *J* = 7.6 Hz, H-5'), 5.88 (1H, d, *J* = 2.1 Hz, H-6), 5.84 (1H, d, *J* = 2.6 Hz, H-8), 4.87 (1H, d, *J* = 11.6 Hz, H-2), 4.47 (1H, d, *J* = 11.0 Hz, H-3); ^{13}C NMR (150 MHz, CD_3OD): δ 196.2, 170.1, 163.9, 163.1, 145.7, 144.9, 128.7, 119.6, 114.8, 114.5, 99.8, 96.7, 95.8, 83.6, 72.3.

Pyrocatechuic acid ***(3)***

White powder, HRESITOFMS *m/z* 153.0180 $[M-H]^-$ (calcd. for $C_7H_5O_4$, 153.0188). ^1H NMR (600 MHz, CD_3OD): δ 7.41 (1H, d, *J* = 2.0 Hz, H-6), 7.34 (1H, dd, *J* = 2.0 Hz and 8.2 Hz, H-4), 6.70 (1H, dd, *J* = 2.7 Hz and 8.2 Hz, H-5); ^{13}C NMR (150 MHz, CD_3OD): δ 174.6, 147.8, 144.0, 129.2, 121.6, 116.34, 113.9.

Gallic acid ***(4)***

White powder, HRESITOFMS *m/z* 169.0143 $[M-H]^-$ (calcd. for $C_7H_5O_5$, 169.0137). ^1H NMR (400 MHz, CD_3OD): δ 7.05 (2H, s, H-2 and H-6); ^{13}C NMR (100 MHz, CD_3OD): δ 169.2, 145.0, 138.1, 120.7, 108.9.

Glucogallin ***(5)***

Yellow powder, HRESITOFMS *m/z* 331.0688 $[M-H]^-$ (calcd. for $C_{13}H_{15}O_{10}$, 331.0665). ^1H NMR (400 MHZ, CD_3OD): δ *glucose moiety* 5.64 (1H, d, *J* = 8.2 Hz, H-1'), 3.85 (1H, dd, *J* = 11.9 Hz and 1.6 Hz, H-6'α), 3.68 (1H, dd, *J* = 12.3 Hz and 4.8 Hz, H-6'β), 3.30 - 3.46 (4H, m, H-2', H-3', H-4', and H-5'), *galloyl moiety* 7.12 (2H, s, H2 and H6); ^{13}C NMR (100 MHz, CD_3OD): δ 165.7, 145.2, 139.0, 119.4, 109.2, 94.6, 77.5, 76.8, 72.8, 69.7, 60.9.

β-Sitosterol ***(6)***

Colorless needle crystals, HREIMS *m/z* 414.9071 $[M]^+$ (indicating a molecular formula $C_{29}H_{50}O$). ^1H NMR (400 MHz, $CDCl_3$): δ 5.35 (1H, t, *J* = 2.7 Hz, H-5), 5.12 - 4.99 (2H, m, H-22 and H-23), 3.59 - 3.48 (1H, m, H-3), 2.17 - 2.31 (1H, m, H-20), 2.06 - 1.81 (10H, m), 1.71 - 1.44 (9H, m), 1.43 - 1.35 (4H, m), 1.13 - 1.07 (3H, m), 1.06 - 0.91 (6H, m, H-19 and H-29), 0.86 - 0.77 (9H, m, H24, H-26, and H-27), 0.73- 0.67 (3H, m, H-28); ^{13}C NMR (100 MHz, $CDCl_3$): δ 140.8, 121.8, 71.8, 56.8, 56.1, 50.3, 50.2, 45.9, 42.4, 42.3, 39.8, 37.3, 36.5, 36.2, 34.0, 31.9, 31.7, 29.2, 28.3, 26.1, 24.3, 23.1, 21.1, 19.9, 19.4, 19.1, 18.8, 12.0, 11.9.

1.2.5 Antioxidant activity

The antioxidant activity of fractions and isolated compounds from *M. casturi* bark was analysed using 1,1-diphenyl-2-picrylhydrazyl (DPPH) method. The diluted working solutions of the fractions, isolated compounds and trolox (positive control) were prepared in methanol. The final concentration of fractions

and isolated compounds are 100 μg/mL and 10 μM, respectively. The reduction of the DPPH was followed by monitoring the decrease in absorbance (Abs) at 545 nm (Kato et al., 2016).

1.2.6. Antileukemic activity

CCK-8 assay: HL-60 cells were obtained from DS Pharma Biomedical Co., Ltd., (Osaka, Japan) and cultured in RPMI 1640 media (Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotics, penicillin-streptomycin (Gibco®, Life Technologies, Thermo Fisher Scientific Inc., MA, USA). Cells were maintained at 37ºC under a humidified atmosphere of 5% CO_2. Cell counting kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). The cells (2.5×10^4 cells/mL) were seeded in 96-well plates. After 24 h, sample solutions were added. Following 48-h incubation, CCK-8 solution (10 μL) was added, and the plates were incubated for an additional 4 h. Visible absorption (490 nm) was measured using a microplate reader (Kakumu et al., 2014; Pardede et al., 2016).

1.2.7. UPLC-ESITOFMS procedures

The samples were dissolved in DMSO/H_2O (1/1) at 20 mg/mL and filtered through 0.45 μm membrane filter (ADVANTEC®, Japan), and an aliquot (5 μL) of the sample was injected in the UPLC. Analysis was carried out by the Waters UPLC system (Aquity UPLC XevoQTof), using a UPLC BEH C_{18} analytical column (1.7 μm, 2.1 × 100 mm). The mobile phase contained solvent A (1% v/v

AcOH in distilled water) and solvent B (acetonitrile). The liner gradient system employed was: 0-30 min 90% solvent A to 70% solvent A and 10% solvent B to 30% solvent B; kept for 5 min; 35-45 min 70% solvent A to 50% solvent A and 30% solvent B to 50% solvent B. The column eluate was monitored at 260 nm UV absorbance. Negative mode was employed in ESITOFMS

1.3. Results and Discussion

1.3.1. Isolation of chemical components from bark of Mangifera casturi

Methanol extract (95.4 g) of the bark of *Mangifera casturi*, followed by solvent partition (*n*-Hex, EtOAc and *n*-BuOH) gave the *n*-Hex fraction (6.4 g), EtOAc fraction (23.0 g) and *n*-BuOH fraction (26.8 g).

The *n*-Hex and EtOAc fraction were separated by CC on silica gel (SiO_2) and purified using PTLC and recrystallization, to yield six compounds. Compound **1 - 5** were isolated from EtOAc fraction and compound **6** was isolated from *n*-Hex fraction. The chemical structures of the six isolated compounds were elucidated by their spectroscopic analysis, MS and literature data.

Compound **2** was isolated as yellow crystals and its molecular formula was established as $C_{15}H_{12}O_7$ from HRESITOFMS *m/z* 303.0479 $[M-H]^-$ (calcd. for $C_{15}H_{11}O_7$, 303.0505). ^1H NMR spectrum of **2** showed the presence of two *meta*-coupled doublet proton on the A ring at δ_H 5.84 (1H, d, J = 2.6 Hz, δ_C 95.8) and 5.88 (1H, d, J = 2.1 Hz, δ_C 96.7), which were assigned to H-8 and H-6, respectively. The remaining aromatic protons at δ_H 6.84 (1H, dd, J = 8.2 and 2.0 Hz, δ_C 119.6), 6.80 (1H, d, J = 7.6 Hz, δ_C 114.7) and 6.97 (1H, d, J = 1.9 Hz, δ_C 114.5) were assigned to H-6', H-5' and H-2', respectively. The ^{13}C NMR spectrum

displayed 11 carbon signals, 1 carbonyl carbon signal at δ_C 196.2 (C-4) (**Figure 4** and **Table 1**). The HRESITOFMS, ^{1}H and ^{13}C NMR data confirmed that **2** were taxifolin (Rusak et al., 2005; Andersen and Markham, 2006; Bahia et al., 2010).

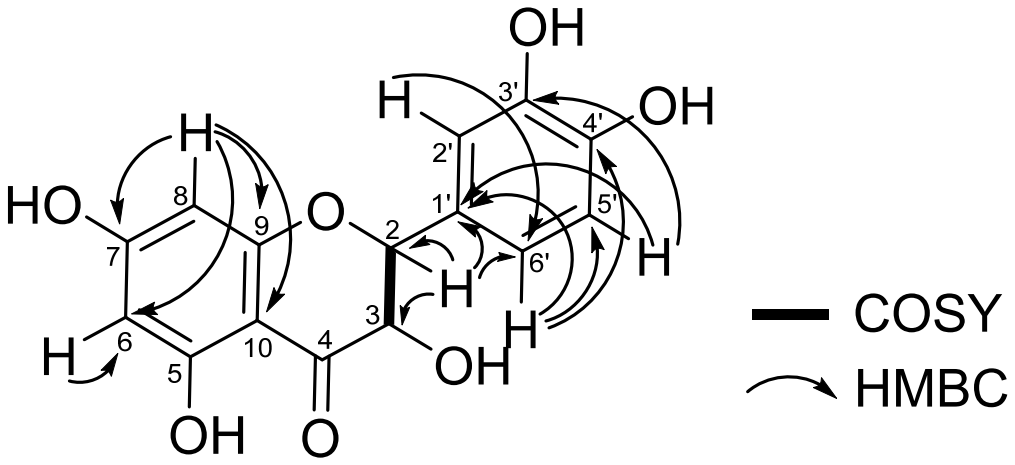


Figure 4. Key ^{1}H - ^{1}H COSY and HMBC correlation of compound **2**.

Table 1. ^{1}H and ^{13}C NMR data of compound **2**.

No	Compound **2** (CD_3OD)		Taxifolin (CD_3OD)*	
	δ_C	δ_H	δ_C	δ_H
2	83.6	4.87, d, *J* = 11.6 Hz	85.1	4.92, d, *J* = 11.0 Hz
3	72.3	4.47, d, *J* = 11.0 Hz	73.6	4.50, d, *J* = 11.0 Hz
4	196.2		198.4	
5	163.1		164.3	
6	96.7	5.88, d, *J* = 2.1 Hz	97.3	5.91, d, *J* = 2.0 Hz
7	170.1		168.7	
8	95.8	5.84, d, *J* = 2.6 Hz	96.3	5.89, d, *J* = 2.0 Hz
9	163.9		164.5	
10	99.8		101.8	
1'	128.7		129.8	
2'	114.5	6.97, d, *J* = 1.9 Hz	115.9	6.97, d, *J* = 2.0 Hz
3'	144.9		146.3	
4'	145.7		147.1	
5'	114.8	6.80, d, *J* = 7.6 Hz	116.1	6.81, d, *J* = 8.0 Hz
6'	119.6	6.84, d, *J* = 2.0, 8.2 Hz	120.9	6.86, dd, *J* = 2.0, 8.0 Hz

*Bahia et al., 2010. *Quim Nova 33*:1297-1300.

According to multiplicities in ^{1}H and ^{13}C NMR spectra of compounds **1**, **3**, **4** and **5**, the compounds skeleton was similar (**Figure 5** and **Table 2**). Compound **4** was isolated as white powder and its molecular formula was established as $C_7H_6O_5$ from HRESITOFMS for the peak at *m/z* 169.0143 $[M-H]^-$ (calcd. for $C_7H_5O_5$, 169.0137). The ^{1}H NMR of compound **4** showed a signal of aromatic proton at δ_H 7.05 (2H, s), which were assigned to H-2 and H-6. The ^{13}C NMR displayed a carboxylic group at δ_C 169.2 and 6 aromatic carbons at δ_C 145 (C-3 and C-5), 138.1 (C-4), 120.7 (C-1) and 108.9 (C-2 and C-6). The HRESITOFMS ^{1}H and ^{13}C NMR data confirmed that **4** were gallic acid (Mahajan and Nandini, 2010; Gangadhar et al., 2011). An additional methyl and glucose moieties to the carboxylic group in the compound **4** determined compound **1** as methyl gallate and **5** as glucogallin, respectively (Majeed at al., 2009; Mahajan and Nandini, 2010; Hisham et al., 2011; Liu et al., 2012). Futhermore, absence of the hydroxyl

group at (C-4 and C-5) and appear hydroxyl group at C-2 in the compound **4** determined compound **3** as pyrocatechuic acid (Benny et al., 2010; George at al., 2011).

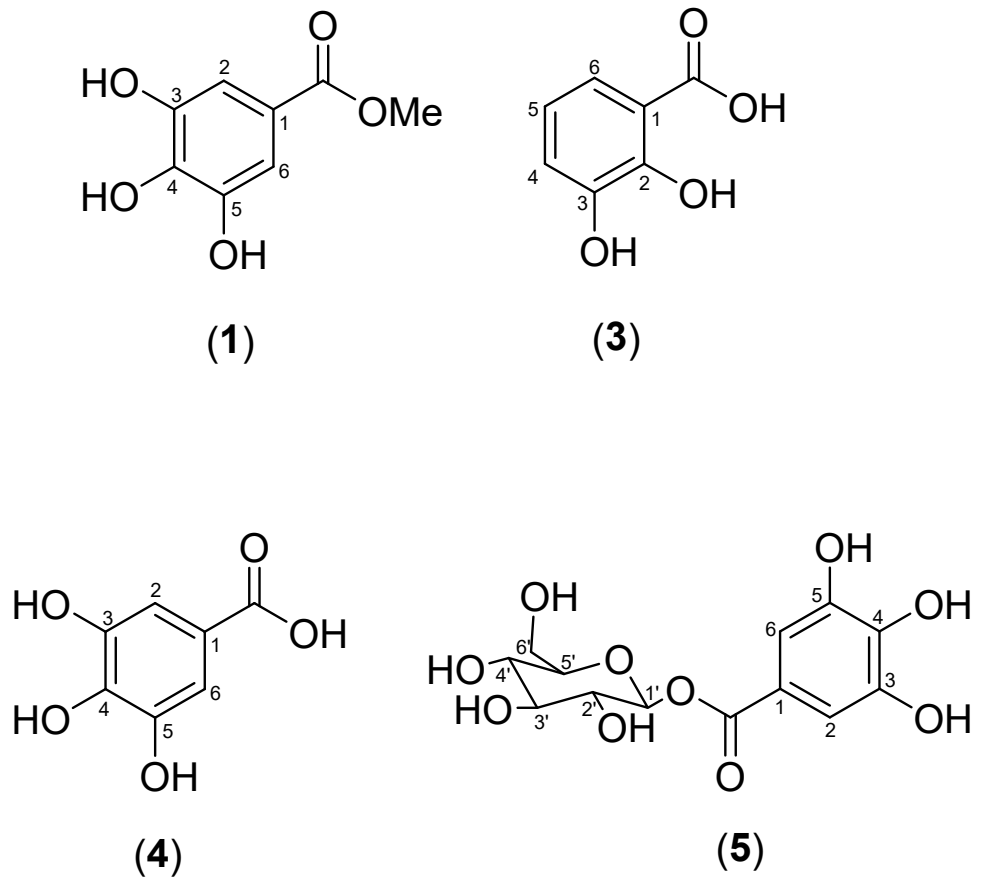


Figure 5. Chemical structures of methyl gallate (**1**), pyrocatechuic acid (**3**), gallic acid (**4**) and glucogallin (**5**).

Table 2. ^{1}H and ^{13}C NMR (solvent: CD_3OD) of methyl gallate (**1**), pyrocatechuic acid (**3**), gallic acid (**4**) and glucogallin (**5**).

No	Methyl Gallate (**1**)		Pyrocatechuic acid (**3**)		Gallic acid (**4**)		Glucogallin (**5**)	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	120.1		147.8		120.7		119.4	
2	108.7	6.99, s	116.2		108.9	7.05, s	109.2	7.12, s
3	145.2		113.8		145		145.2	
4	138.4		121.6	7.34, dd, *J* = 2.0, 8.2 Hz	138.1		139	
5	145.2		129.1	6.70, dd, *J* = 2.7, 8.2 Hz	145		145.2	
6	108.7	6.99, s	144	7.41, d, *J* = 2.0 Hz	108.9	7.05, s	109.2	7.12, s
C=O	167.7		174.5		169.2		165.7	
OMe	50.6	3.77, s						
1'							94.6	5.64, d, *J* = 8.2 Hz
2'							72.8	3.46 - 3.30, m
3'							76.8	3.46 - 3.30, m
4'							69.7	3.46 - 3.30, m
5'							77.5	
6' β							60.9	3.68, dd, *J* = 11.9, 1.6 Hz
6' α								3.85, dd, *J* = 11.9 Hz and 1.6 Hz

Compound **6** was isolated as colorless needle crystals. On the TLC, the spot of compound **6** was inactive at UV lamp 254 and 365 nm, which was primary characteristic of steroids compounds. The molecular formula was established as $C_{29}H_{50}O$ from HREIMS *m/z* 414.9071 $[M]^+$. The spectral data of compound **6** were identical to those previously reported (Kamboj and Saluja, 2011; Chaturvedula and Prakash, 2012; Mahdavi, 2014), **6** was confirm as as β-sitosterol (**Figure 6** and **Table 3**).

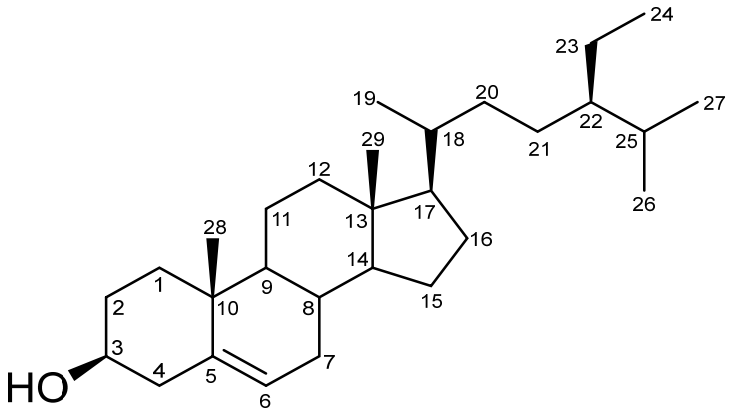


Figure 6. Chemical structure of compound (**6**).

Table 3. ^{1}H and ^{13}C NMR of compound (**6**).

No	β-sitosterol ($CDCl_3$)*	Compound (**6**) ($CDCl_3$)	
	δ_C	δ_C	δ_H
1	37.5	39.8	2.06 – 1.07, m
2	31.9	31.7	2.06 – 1.07, m
3	72.0	71.8	3.59 – 3.48, m
4	42.5	42.4	2.06 – 1.07, m
5	140.9	140.8	
6	121.9	121.8	5.35, t, J = 2.7 Hz
7	32.1	31.9	2.06 – 1.07, m
8	32.1	34.0	2.06 – 1.07, m
9	50.3	50.3	2.06 – 1.07, m
10	36.7	37.3	
11	21.3	21.1	2.06 – 1.07, m
12	39.9	42.3	2.06 – 1.07, m
13	42.6	45.9	
14	56.9	56.8	2.06 – 1.07, m
15	26.3	24.3	2.06 – 1.07, m
16	28.5	28.3	2.06 – 1.07, m
17	56.3	56.1	2.06 – 1.07, m
18	36.3	36.5	2.06 – 1.07, m
19	19.2	19.1	1.06 – 0.91, m
20	34.2	36.2	2.31 – 2.17, m
21	26.3	26.1	2.06 – 1.07, m
22	46.1	50.2	5.12 – 4.99, m
23	23.3	23.1	5.12 – 4.99, m
24	12.2	12.0	0.86 – 0.77, m
25	29.4	29.2	2.06 – 1.07, m
26	20.1	19.9	0.86 – 0.77, m
27	19.6	19.4	0.86 – 0.77, m
28	19.0	18.8	0.73 – 0.67, m
29	12.0	11.9	1.06 – 0.91, m

*Chaturvedula and Prakash, 2012. *Int. Curr. Pharm. J. 1*: 239-242.

All compounds were isolated from bark of *Mangifera casturi* for the first time. The chemical structures of isolated compounds from bark of *Mangifera casturi* were given in **Figure 7**.

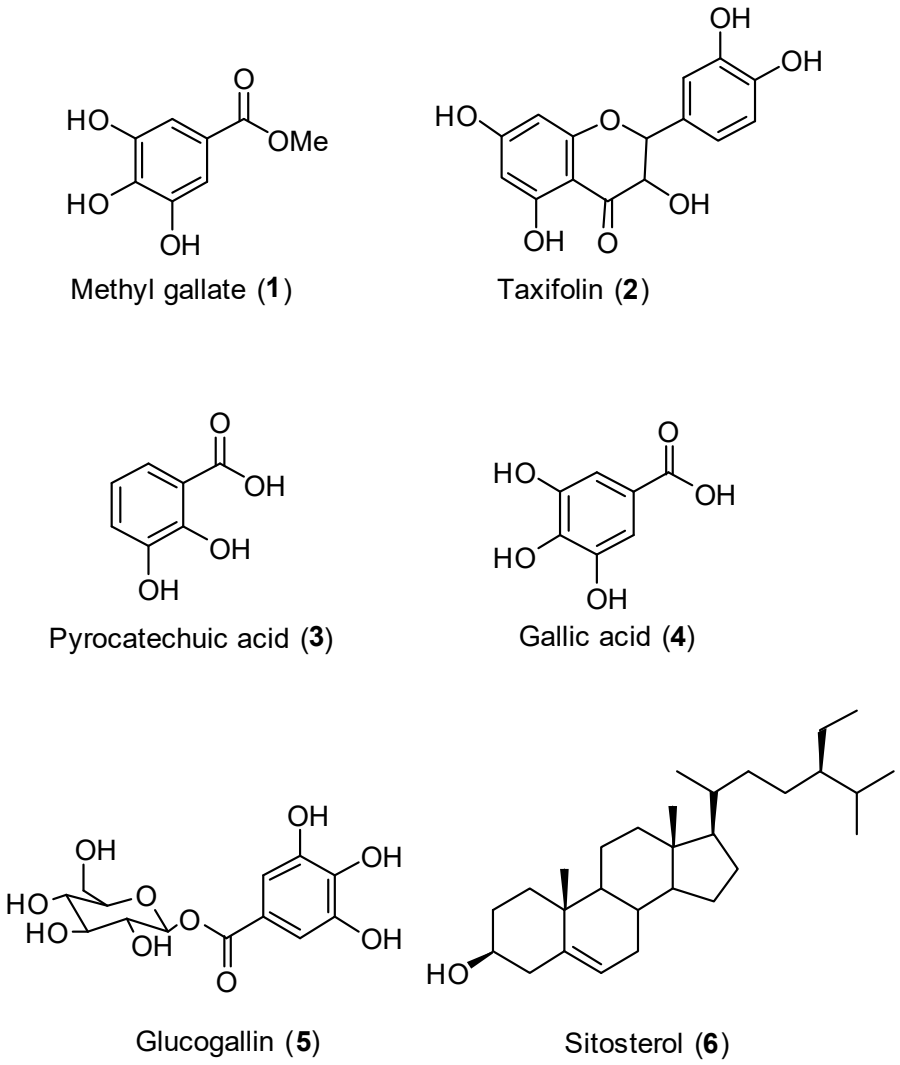


Figure 7. Chemical structures of isolated compounds from bark of *Mangifera casturi.*

1.3.2. Antioxidant and antileukemic activity

The methanol extract of *Mangifera casturi* bark was partitioned successively with *n*-Hex, EtOAc and *n*-BuOH. Antioxidant and antileukemic activity of the each fraction were evaluated. EtOAc fraction had the highest antioxidant activity (96.4%) and also showed highest activity against human leukemia HL-60 with cell

survival rate of 52.6% (**Figures 8 and 9**). Furthermore, the antioxidant and antileukemic activities of isolated compounds were confirmed.

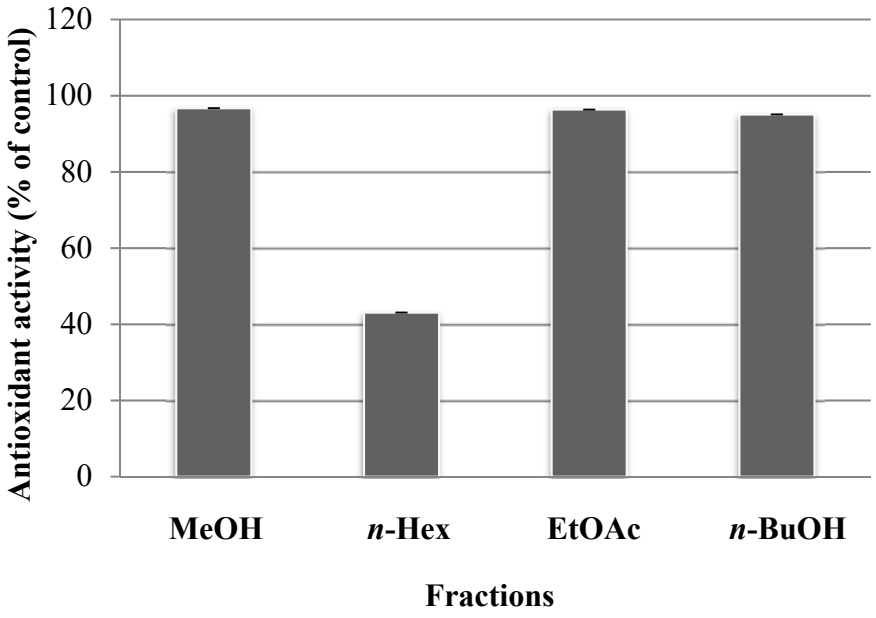


Figure 8. Antioxidant activity of fractions from *Mangifera casturi* (Means ± SEMs, $n = 3$, 100 µg/mL).

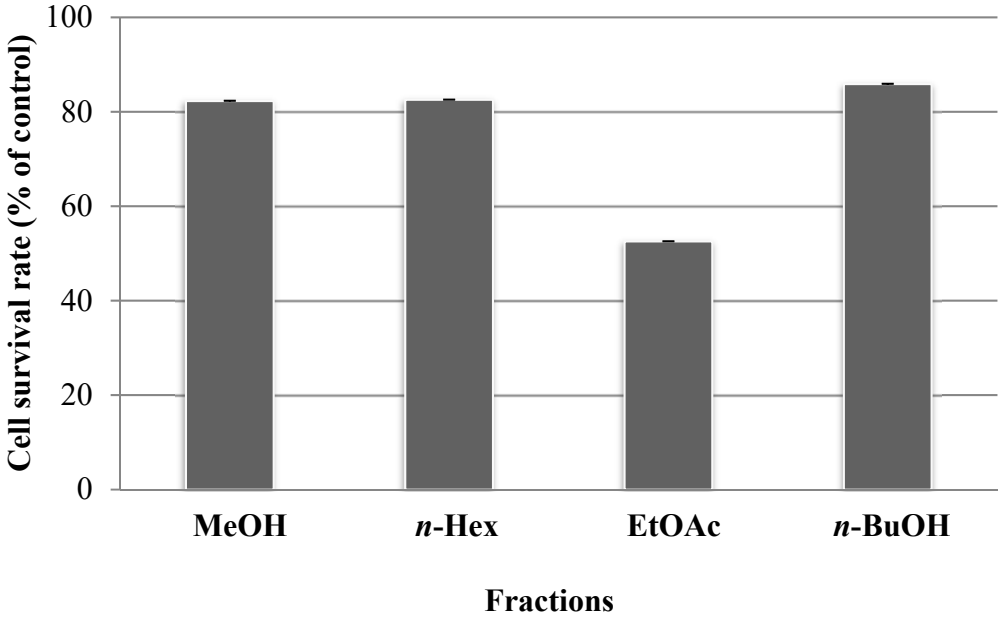


Figure 9. Antileukemic activity of fractions Mangifera casturi (Means ± SEMs, $n = 3$, 100 µg/mL).

The hydroxyl groups are important for free radical scavenging efficiency. In particular, the hydroxyl group at the *para*-position to the carboxylic group appears essential to maintain the scavenging activity (Lu et al., 2006), based on these data, the hydroxyl group at the *para*-position to the carboxylic group in the gallic acid (**4**) showed high antioxidant activity when compared to pyrocatechuic acid (**3**). Furthermore, additional methyl or glucose group into a carboxylic moiety of gallic acid (**4**) decreased antioxidant activity. This can be seen in gallic acid (**4**) when compared with methyl gallate (**1**) and glucogallin (**5**), indicating that the presence of methyl or glucose moiety reduces antioxidant activity (**Figure. 10**).

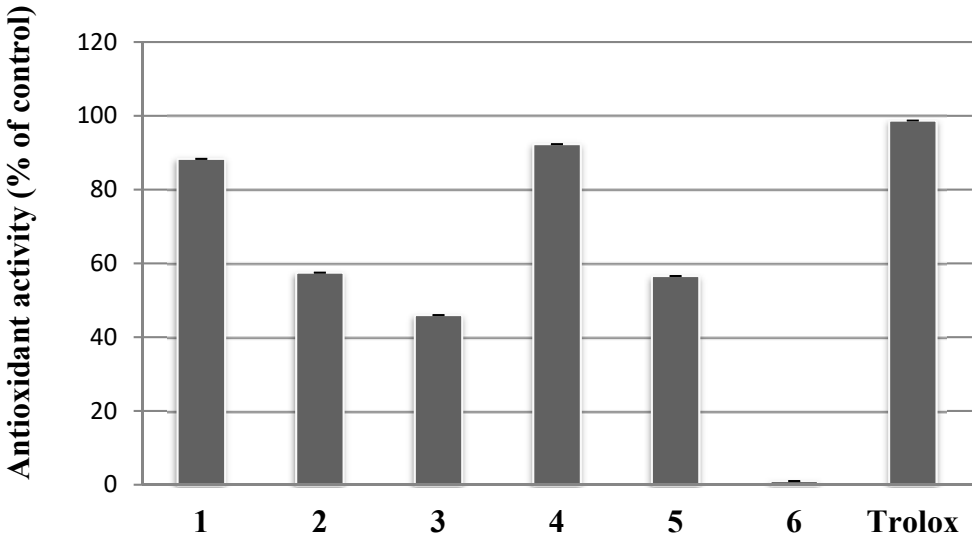


Figure 10. Antioxidant activity of isolated compounds from bark of *Mangifera casturi* (Means ± SEMs, *n* = 3, 10 μM). Methyl gallate (**1**), taxifolin (**2**), pyrocatechuic acid (**3**), gallic acid (**4**), glucogallin (**5**) and β-sitosterol (**6**).

In flavonoids, the C2 - C3 double bond with a 4-oxo functional group in the C ring is responsible for its antioxidant activity, this is through electron delocalization from the B ring. The antioxidant activity of taxifolin (**2**) is 57.5% because it lacks the C2 – C3 double bond, although taxifolin (**2**) has potential for maximum radical scavenging activity in the 3- and 5-hydroxyl group in A and C ring (Dai and Mumper, 2010).

We evaluated the antileukemic activity of isolated compounds against HL-60 cells using the CCK-8 assay method. The isolated compounds, methyl gallate (**1**), taxifolin (**2**), pyrocatechuic acid (**3**), gallic acid (**4**), glucogallin (**5**) and β-

sitosterol (**6**) at a final concentration of 50 µM exhibited cell survival rate 37.5%, 97.0%, 58.8%, 9.75%, 57.3% and 93.2%, respectively, shown in **Figure 11**.

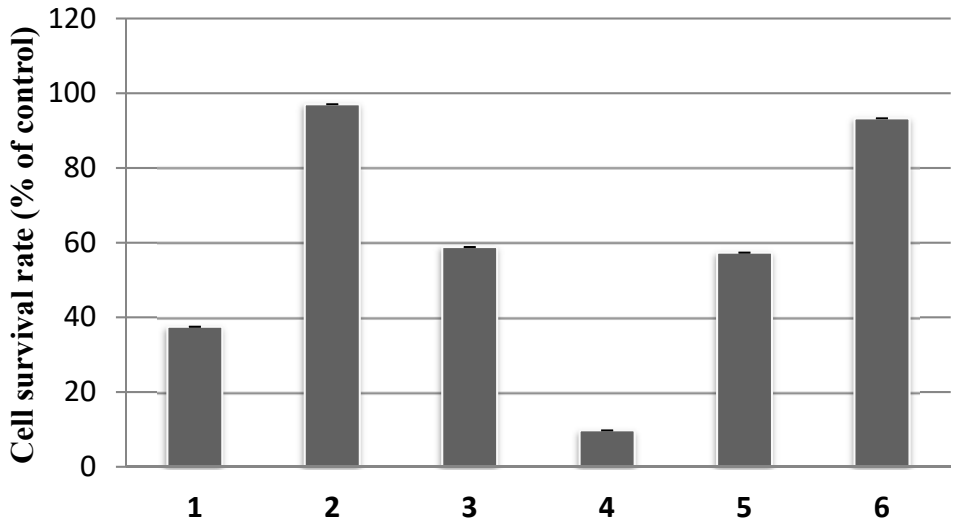


Figure 11. Antileukemic activity of isolated compounds from bark of *Mangifera casturi* (Means ± SEMs, *n* = 6, 50 µM). Methyl gallate (**1**), taxifolin (**2**), pyrocatechuic acid (**3**), gallic acid (**4**), glucogallin (**5**) and β-sitosterol (**6**).

Comparing literature data, cell survival rate of quercetin was 29.7% (Kakumu et al., 2014) and that of taxifolin (**2**) was 97.0% in human leukemia HL-60 cells; it was observed that the structure of quercetin and taxifolin (**2**) was similar; the major difference was the absence of C2 - C3 double bond in the structure of taxifolin (**2**). This makes it ineffective against human leukemia HL-60 cells (Rusak et al., 2005).

The addition of a methyl and glucose group to the carboxylic moiety of gallic acid decreased its antileukemic activity (Kakumu et al., 2014). Gallic acid (**4**) strongly inhibited the cell proliferation with cell survival rate of 9.71%, when compared with methyl gallate (**1**) (37.5%) and glucogallin (**5**) (57.3%).

1.3.3. UPLC-ESITOFMS analysis

Analysis of the chemical components from EtOAc fraction of *M. casturi* bark was carried out using UPLC-ESITOFMS. The chemical components were assigned as $[M-H]^-$ ions in **Figure 12**. The retention time of each compound in the EtOAc fraction were assigned 5.23, 5.74, 11.05, 15.72, and 21.95 min for glucogallin (**5**), gallic acid (**4**), pyrocatechuic acid (**3**), methyl gallate (**1**) and taxifolin (**2**), respectively. The HRESITOFMS spectrum of each retention time of compounds isolated from bark of *Mangifera casturi* were given in **Figure 13**.

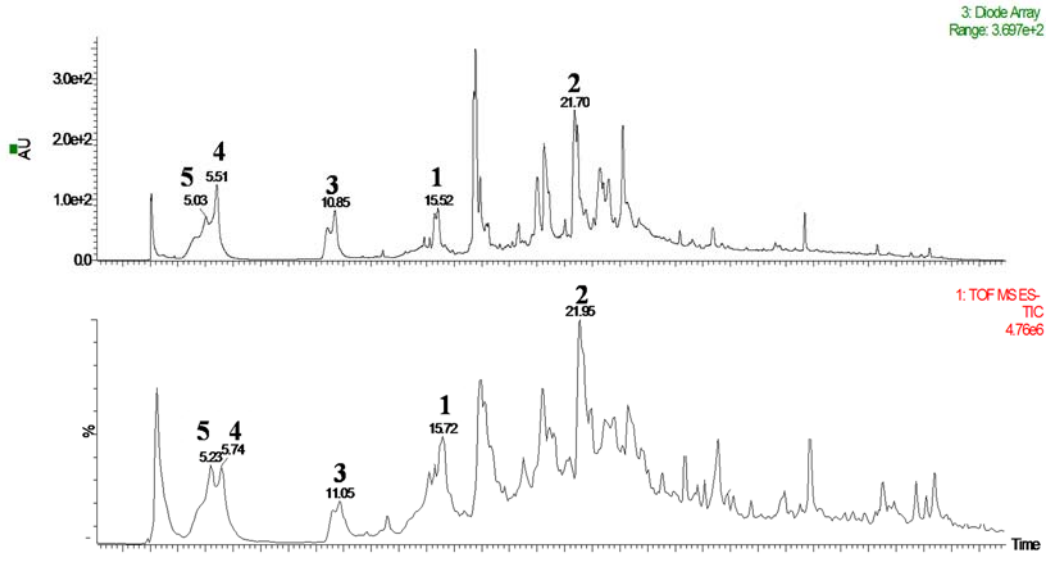


Figure 12. UPLC-ESITOFMS chromatogram of the EtOAc fraction of *Mangifera casturi* bark. Methyl gallate (**1**), taxifolin (**2**), pyrocatechuic acid (**3**), gallic acid (**4**) and glucogallin (**5**).

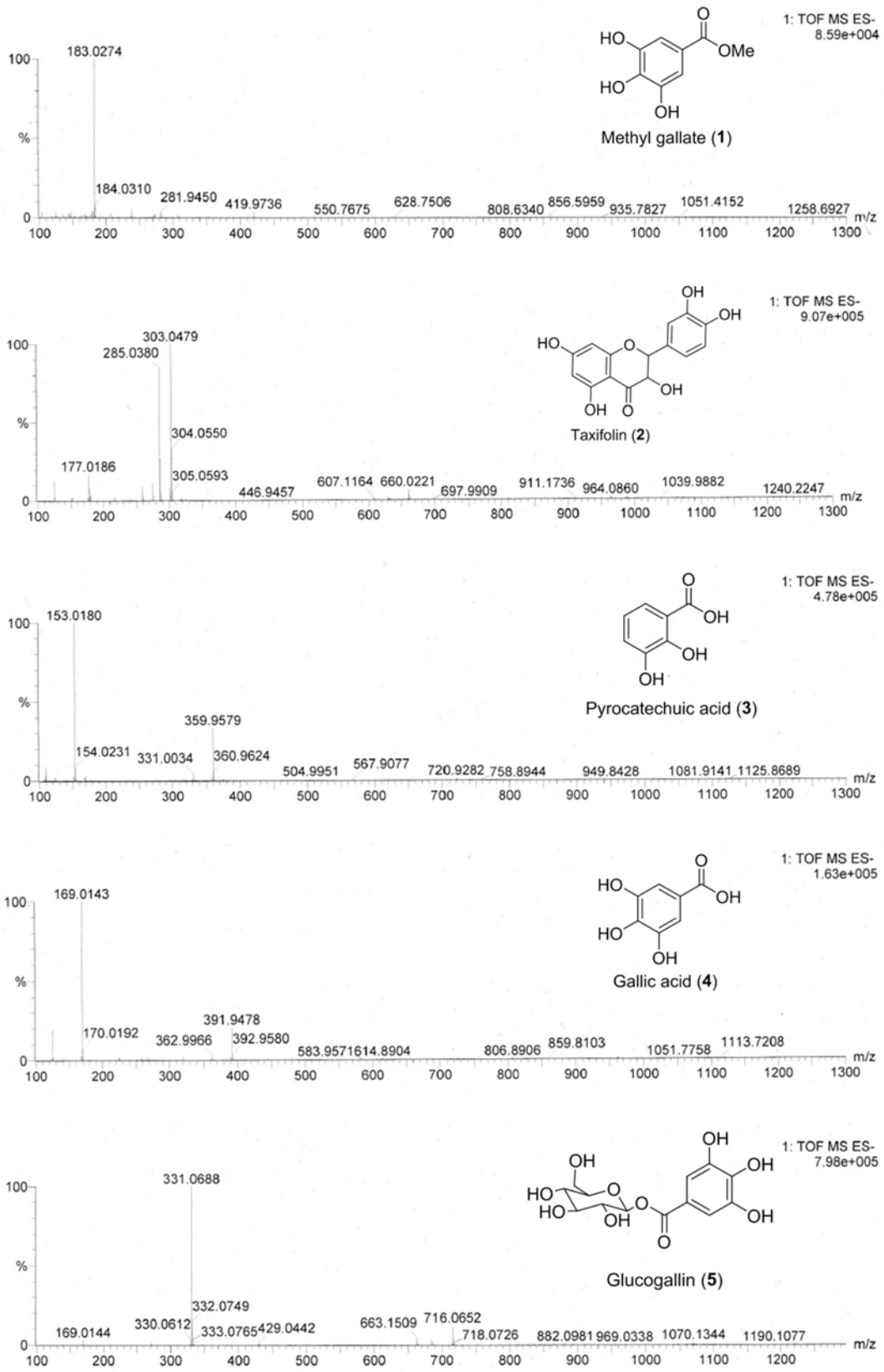


Figure 13. The HRESITOFMS spectrum of each retention time of compounds isolated from bark of *Mangifera casturi*.

1.4. Conclusions

In summary, methyl gallate (**1**), taxifolin (**2**), pyrocatechuic acid (**3**), gallic acid (**4**), glucogallin (**5**) and β-sitosterol (**6**) were isolated from bark of *M. casturi* for the first time (**Figure 14**). The antioxidant and antileukemic activities of isolated compounds were evaluated. The presence and position of hydroxyl, methyl and glucose group in the isolated compounds affects their antioxidant and antileukemic activities. *M. casturi* bark is a good source of gallic acid and the derivatives.

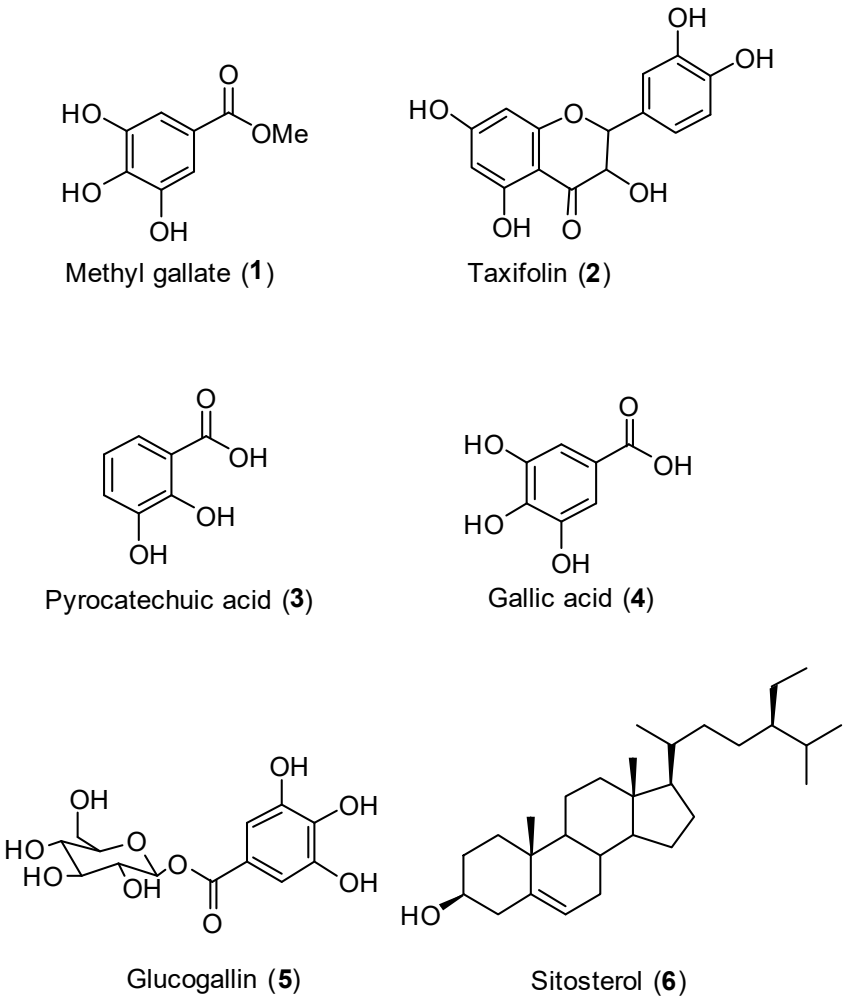


Figure 14. Chemical structures of isolated compounds from bark of *Mangifera casturi.*

References

Afifa, K., Kamruzzaman, M., Mahfuza, I. 2014. A comparison with antioxidant and functional properties among five mango (*Mangifera indica* L.) varieties in Bangladesh. *Int. Food Res. J. 21*:1501-1506.

Ahmad, S., Sukari, M. A., Ismail, N. 2015. Phytochemicals from *Mangifera pajang* Kosterm and their biological activities. *Compl. Alter. Med.* 15:83.

Andersen, Ø. M., Markham, K. R. 2006. Flavonoids chemistry, biochemistry and applications. Taylor & Francis Group, New York, pp 67-73.

Bahia, M.V., David, J. P., David, J. M. 2010. Occurrence of biflavones in leaves of *Caesalpinia pyramidalis* specimens. *Quim Nova 33*:1297-1300.

Benny, P. J., Shibumon., Sunny, K., Cincy, G. 2010. 2,3-Dihydroxybenzoic acid: An effective antifungal agent isolated from *Flacourtia inermis* fruit. *Int. J. Pharm. Clin. Res. 2*:101-105.

Chaturvedula, V. S. P., Prakash, I. 2012. Isolation of stigmasterol and β-sitosterol from the dichloromethane extract of *Rubus suavissimus*. *Int. Curr. Pharm. J. 1*: 239-242.

Dai, J., Mumper, R. J. 2010. Plant phenolics: extraction, analysis, and their antioxidant and anticancer properties. *Molecules 15*:7313.

Fakhrudin, N., Putri, P. S., Sutomo., Wahyuono, S. 2013. Antiinflamatory activity of methanolic extract of *Mangifera casturi* in thioglycollate-induced leukocyte migration on mice. *Trad. Med. J. 18*:151-156.

Gangadhar, M., Bhavana, P., Sunil, Y., Datta, S. 2011. Isolation and characterization gallic acid from *Terminilia bellerica* and its effect on carbohydrate regulatory system in vitro. *Int. J. Res. Ayurveda Pharm. 2*:559-562.

George, S., Benny, P. J., Sunny, K., Cincy, G. 2011. Antibiotic activity of 2,3-dihydroxybenzoic acid isolated from *Flacourtia inermis* fruit against multidrug resistant bacteria. *Asian J. Pharm. Clin. Res. 4*:126-130.

Hisham, D. M. N., Lip, J. M., Noh, J. M. 2011. Identification and isolation of methyl gallate as a polar chemical marker from *Labisia pumila Benth*. *J. Trop. Agric. Food Sci. 39*:279-284.

Kakumu, A., Ninomiya, M., Efdi, M., Adfa, M., Hayashi, M., Tanaka, K., Koketsu, M. 2014. Phytochemical analysis and antileukemic activity of poliphenolic constituents of *Toona sinensis*. *Bioorg. Med. Chem. Lett. 24*:4286-4290.

Kamboj, A., Saluja, A. K. 2011. Isolation of stigmasterol and β-sitosterol from petroleum ether extract of aerial parts of *Ageratum conyzoides* (Asteraceae). *Int. J. Pharm. Sci. 3*:94-96.

Kato, K., Ninomiya, M., Tanaka, K., Koketsu, M. 2016. Effect of functional groups and sugar composition of quercetin derivatives on their radical scavenging properties. *J. Nat. Prod. 79*:1808-1814.

Liu, Y., Gao, L., Liu, L. 2012. Purification and characterization of a novel galloyltransferase involved in catechin galloylation in the tea plant *Camellia sinensis*. *J. Biol. Chem. 6*:1-22.

Lu, Z., Nie, G., Belton, P. S. 2006. Structure activity relationship analysis of antioxidant ability and neuroprotective effect of gallic acid derivatives. *Neurochem. Int. 48*:263-274.

Mahajan, A., Nandini, P. 2010. Simultaneous isolation and identification of phytoconstituents from *Terminalia chebula* by preparative chromatography. *J. Chem. Pharm. Res. 2*:97-103.

Mahdavi, B. 2014. Chemical constituents of the aerial parts of *Etlingera brevilabrum* (Zingiberaceae). *Der. Pharma. Chem. 6*:360-365.

Majeed, M., Bhat, B., Jadhav, A. N. 2009. Ascorbic Acid and Tannins from *Emblica officinalis* Gaertn Fruitss A Revisit. *J. Agric. Food Chem. 57*:220-225.

Meneses, M. A., Caputo, G., Scognamiglio, M. 2015. Antioxidant phenolic compounds recovery from *Mangifera indica* L. by-products by supercritical antisolvent extraction. *J. Food Eng. 163*:45-53.

Pardede, A., Mashita, K., Ninomiya, M., Tanaka, K., Koketsu, M. 2016. Flavonoid profile and antileukemic activity of *Coreopsis lanceolata* flowers. *Bioorg. Med. Chem. Lett. 26*:2784-2787.

Rivera, D. G., Delgado, R., Bougarne, N. 2011. Gallic acid indanone and mangiferin xanthone are strong determinants of immunosuppressive anti-tumour effects of *Mangifera indica* L. bark in MDA-MB231 breast cancer cells. *Cancer Lett. 305*:21-31.

Rodeiro, I., Cancino, L., Gonzales, J. E. 2006. Evaluation of the genotoxic potential of *Mangifera indica* L. extract (Vimang), a new natural product with antioxidant activity. *Food Chem. Toxicol. 44*:1707-1713.

Rusak, G., Gutzeit, O. H., Muller, J. L. 2005. Structurally related flavonoids with antioxidative properties differentially affect cell cycle progression and apoptosis of human acute leukemia cells. *Nutr. Res. 25*:141-153.

Suhartono, E. Viani, E., Rahmadhan, M. A. 2012. Total flavanoid and antioxidant activity of some selected medicinal plants in South Kalimantan of Indonesia. *Asia Pacific Chem. Biol. Environ. Eng. 4*:235-239.

Sutomo., Wahyuono., Rianto., Setyowati. 2013. Isolation and identification of active compound of n-hexane fraction from kasturi (*Mangifera casturi* Kosterm) against antioxidant and immunomodulatory activity. *J. Biol. Sci. 13*:596-604.

Chapter 2

Flavonoid rutinosides from *Cinnamomum parthenoxylon* leaves and their hepatoprotective and antioxidant activity

2.1. Introduction

Cinnamomum parthenoxylon tree belongs to the *Lauraceae* family. It is called kayu gadis and has been used by local people as spices in foods, fragrances, fumigants, and traditional medicines in Indonesia (Wang et al., 2013; Kawatra and Rajagopalan, 2015). The picture of *Cinnamomum parthenoxylon* part is show in **Figure 15.**

a b

Figure 15. *Cinnamomum parthenoxylon* stem (a) and leaves (b).

The species from *Cinnamomum* genus are known to have various biological activities. For instance, *Cinnamomum verum, Cinnamomum loureirii, Cinnamomum burmannii* and *Cinnamomum cassia* showed high inhibitory

activities against various cancer cell lines and are also employed in the treatment of diabetes (Hong et al., 2002; Jia et al., 2009; Unlu et al., 2010; Cao et al., 2010; Daker et al., 2013; Lee et al., 2013). Previous studies indicated that several lignans and phenylpropanoids such as dehydroxycubebin, hinokinin, cubebin, 3-(3,4-methylenedioxyphenyl)-1,2-propanediol and safrole isolated from *Cinnamomum parthenoxylon* woods have been reported to exert antileukemic activity against human leukemia HL-60 and U937 cells (Adfa et al., 2016). The lignans and phenylpropanoids isolated from *Cinnamomum parthenoxylon* wood shown in **Figure 16.**

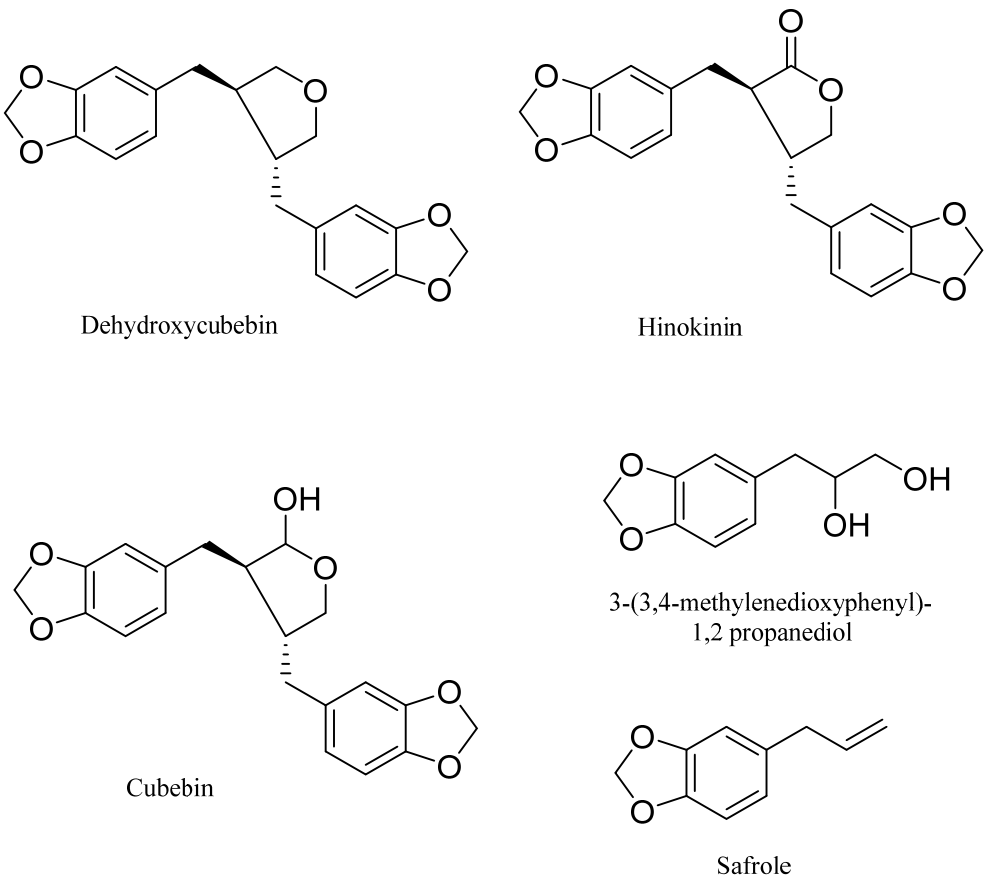


Figure 16. Isolated compounds from *Cinnamomum parthenoxylon* woods.

Thus far, based on literature survey (Wei et al., 2017), no biological activities has been reported on the compounds isolated from *Cinnamomum parthenoxylon* leaves. Flavonoid is an important class of secondary metabolites from plants and possesses a wide spectrum of pharmacological properties, such as antileukemic activity (Ninomiya et al., 2013; Pardede et al., 2016). Flavonoids have received much attention in human health due to its antioxidant activity (Kato et al., 2016), hepatoprotective activity as well as reducing oxidative stress (Kinjo et al., 2006). Hence, the hepatoprotective and antioxidant activity of flavonoid rutinosides isolated from *C. parthenoxylon* leaves was investigated.

2.2. Materials and Methods

2.2.1. General experimental procedures

All solvents and reagents were purchased from the suppliers and used without further purification. MS spectra were obtained using UPLCMS system (Aquity UPLC XevoQTof). ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectra were recorded with a JEOL ECX400 spectrometer with tetramethylsilane as an internal standard. Silica gel column chromatography (CC) was performed on silica gel N-60 (40-50 μm). Thin-layer chromatography (TLC) spots on plates pre-coated with silica gel 60 F_{254} were detected with a UV lamp (254 nm). Fractionations for all CC were based on TLC analyses.

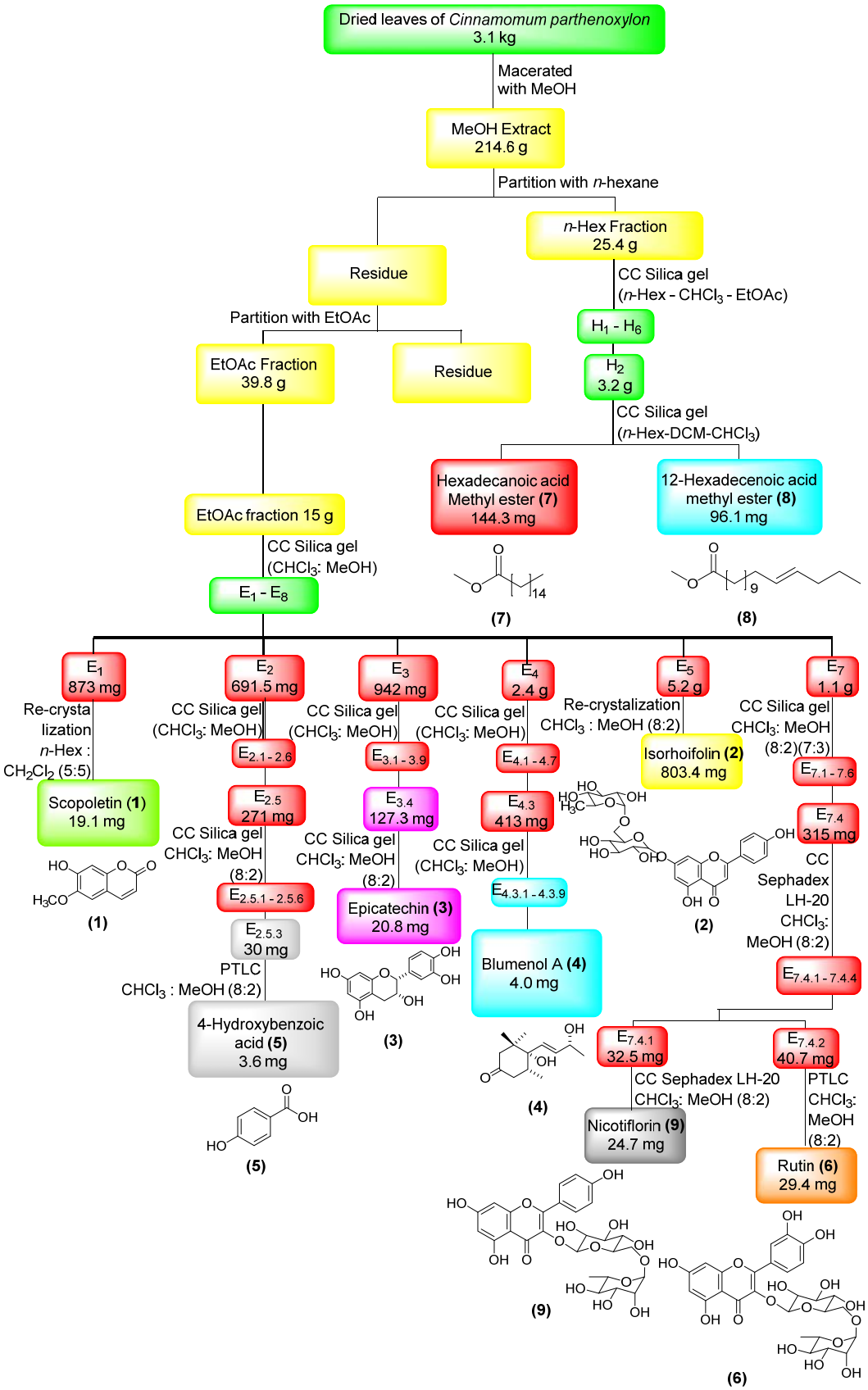
2.2.2. Extraction and isolation chemical constituents from Cinnamomum parthenoxylon leaves

Dried leaves of *Cinnamomum parthenoxylon* (3.1 kg) was macerated with methanol at room temperature. The extract was filtered and evaporated *in vacuo* to yield methanol extract (214.6 g). The methanol extract was suspended in water and partitioned successively with *n*-hexane and EtOAc to yield *n*-hexane fraction (25.4 g) and EtOAc fraction (39.8 g).

A portion of EtOAc fraction (15.0 g) was separated by silica gel CC eluted with $CHCl_3$ - MeOH, which yielded 8 fractions (E_1 - E_8). Compound **1** (19.1 mg) and compound **2** (803.4 mg) was isolated from fraction E_1 (873 mg) and E_5 (5.2 g) by recrystallization, respectively. Fraction E_3 was separated by silica gel CC eluted with $CHCl_3$ - MeOH to give 9 subfractions ($E_{3.1}$ - $E_{3.9}$). $E_{3.4}$ (127.3 mg) was further purified by silica gel CC eluting $CHCl_3$ - MeOH (8: 2), to obtained compound **3** (20.8 mg). Fraction E_4 (2.4 g) was separated by silica gel (CC) eluted $CHCl_3$ – MeOH stepwise manner of polarity to give 7 subfraction ($E_{4.1}$ - $E_{4.7}$). $E_{4.3}$ (413 mg) was further purified by silica gel CC eluting $CHCl_3$ - MeOH, to obtained compound **4** (4.0 mg). Fraction E_2 (691.5 mg) was divided by silica gel CC eluted $CHCl_3$ - MeOH stepwise manner of polarity to give 6 subfraction ($E_{2.1}$ - $E_{2.6}$). $E_{2.5}$ (271 mg) was applied by silica gel CC eluted with $CHCl_3$ - MeOH (8 : 2), to give 6 subfraction ($E_{2.5.1}$ - $E_{2.5.6}$), furthermore subfraction $E_{2.5.3}$ (30 mg) was purified using preparative thin layer chromatography (PTLC) eluted with ($CHCl_3$ - MeOH 8 : 2), to gave compound **5** (3.6 mg). Fraction E_7 (1.1 g) was applied by silica gel CC eluted with $CHCl_3$ - MeOH (8 : 2) (7 : 3), to give 8 subfraction ($E_{7.1}$ – $E_{7.8}$). $SubFr_{7.4}$ was separated using Sephadex LH-20 CC eluting ($CHCl_3$ - MeOH 8 : 2),

to give 4 subfraction ($E_{7.4.1}$ – $E_{7.4.4}$). $SubFrE_{7.4.2}$ was purified by PTLC ($CHCl_3$ - MeOH 8 : 2) to yield compound **6** (29.4 mg). $SubFrE_{7.4.1}$ was purified by Sephadex LH-20 eluting ($CHCl_3$ - MeOH 8 : 2) to yield compound **9** (24.7 mg).

The *n*-hexane fraction was divided by silica gel CC eluting *n*-hexane - $CHCl_3$ - EtOAc step gradient polarity to yield 6 fractions (H_1 to H_6). Fraction H_2 (3.2 g) was further purified by silica gel CC eluting *n*-hexane - CH_2Cl_2 - $CHCl_3$ stepwise manner of polarity to afford compound **7** (144.3 mg) and compound **8** (96.1 mg), respectively (**Scheme 2**).



Scheme 2. Isolation scheme of *Cinnamomum parthenoxylon* leaves extract.

2.2.3. Spectral data of isolated compounds

*Scopoletin (**1**)*

Pale yellow crystals, HRESITOFMS *m/z* 191.0328 $[M-H]^-$ (calcd. for $C_{10}H_7O_4$, 191.0344). ^1H NMR (400 MHz, $CDCl_3$): δ 7.84 (1H, d, *J* = 9.6 Hz, H-4), 7.19 (1H, s, H-5), 6.79 (1H, s, H-8), 6.18 (1H, d, *J* = 9.6 Hz, H-3), 3.90 (3H, s, OCH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 160.5, 151.0, 150.3, 145.1, 143.8, 112.49, 111.3, 109.1, 102.9, 55.9.

*Isorhoifolin (**2**)*

Yellow powder, HRESITOFMS *m/z* 577.1555 $[M-H]^-$ (calcd. for $C_{27}H_{29}O_{14}$, 577.1557). ^1H NMR (400 MHz, DMSO): δ 7.95 (2H, d, *J* = 9.2 Hz, H-2' and H-6'), 6.95 (2H, d, *J* = 9.2 Hz, H-3' and H-5'), 6.88 (1H, s, H-3), 6.80 (1H, d, *J* = 2.3 Hz, H-8), 6.38 (d, *J* = 1.8 Hz, H-6), 5.24 (1H, d, *J* = 7.3 Hz, H-1'''), 5.14 (1H, s, H-1''), 5.36, 5.18, 4.75, 4.69, 4.50 (6H, sugar hydroxyls), 3.21 – 3.77 (10H, m, sugar protons), 1.21 (3H, d, *J* = 6.4 Hz, H-6'''); ^{13}C NMR (100 MHz, DMSO): δ 182.5, 164.8, 163.1, 161.9, 161.6, 157.5, 129.1, 121.1, 116.6, 106.0, 103.7, 101.0, 99.8, 98.3, 95.0, 77.5, 76.8, 72.4, 71.0, 70.9, 70.6, 70.1, 68.9, 61.0, 18.6.

*Epicatechin (**3**)*

Yellow powder, HRESITOFMS *m/z* 289.0714 $[M-H]^-$ (calcd. for $C_{15}H_{13}O_6$, 289.0712). ^1H NMR (400 MHz, Acetone-d_6): δ 7.06 (1H, d, *J* = 1.8 Hz, H-2'), 6.84 (1H, dd, *J* = 8.5 Hz and 1.8 Hz, H-6'), 6.79 (1H, d, *J* = 8.2 Hz, H-5'), 6.03 (1H, d, *J* = 2.3 Hz, H-6), 5.92 (1H, d, *J* = 2.3 Hz, H-8), 4.88 (1H, s, H-2), 4.21 (1H, s, H-3), 2.87 (1H, dd, *J* = 16.7 Hz and 4.6 Hz, H-4β), 2.74 (1H, dd, *J* = 16.9

Hz and 3.2 Hz, H-4α); ^{13}C NMR (100 MHz, Acetone-d_6): δ 156.8, 156.7, 156.3, 144.6, 144.5, 131.4, 118.5, 114.7, 114.5, 99.0, 95.3, 94.9, 78.6, 66.1, 28.8.

*Blumenol A (**4**)*

Pale yellow powder, HRESITOFMS *m/z* 225.1479 $[M-H]^-$ (calcd. for $C_{13}H_{21}O_3$, 225.1491). ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.83 (1H, dd, *J* = 15.8 Hz and 5.5 Hz, H-8), 5.70 (1H, d, *J* = 16.9 Hz, H-7), 4.44 (1H, quint, H-9), 2.84 (1H, d, *J* = 13.3 Hz, H-2 ax), 2.40 1(H, d, *J* = 12.8 Hz, H-4 ax), 2.29 (1H, m, H-5), 2.13 (1H, dd, *J* = 13.4 Hz and 2.1 Hz, H-4 eq), 1.92 (H, dd, *J* = 13.8 Hz and 2.3 Hz, H-2 eq), 1.33 (3H, d, *J* = 6.4 Hz, H-10), 0.97 (3H, s, H-11), 0.94 (3H, s, H-12), 0.90 (3H, d, *J* = 6.6 Hz, H-13) ^{13}C NMR (100 MHz, $CDCl_3$): δ 211.3, 135.2, 131.9, 77.3, 68.4, 51.5, 45.2, 42.6, 36.4, 24.5, 24.4, 23.9, 16.0.

*4-Hydroxybenzoic acid (**5**)*

White powder, HRESITOFMS *m/z* 137.0265 $[M-H]^-$ (calcd. for $C_7H_5O_3$, 137.0239). ^{1}H NMR (400 MHz, Acetone-d_6): δ 7.92 (2H, d, *J* = 9.2 Hz, H-2 and H-6), 6.91 (2H, d, *J* = 9.2 Hz, H-3 and H-5); ^{13}C NMR (100 MHz, Acetone-d_6): δ 167.8, 162.5, 132.7, 123.1, 115.9.

*Rutin (**6**)*

Yellow powder, HRESITOFMS *m/z* 609.1476 $[M-H]^-$ (calcd. for $C_{27}H_{29}O_{16}$, 609.1456). ^{1}H NMR (400 MHz, CD_3OD): δ 7.67 (1H, d, *J* = 2.3 Hz, H-2'), 7.62 (1H, dd, *J* = 8.0 Hz, and 2.3 Hz, H-6'), 6.87 (1H, d, *J* = 8.7 Hz, H-5'), 6.40 (1H, d, *J* = 2.3 Hz, H-8), 6.21 (1H, d, *J* = 2.3, H-6), 5.11 (1H, d, *J* = 7.8 Hz, H-1''), 4.52

(1H, d, *J* = 1.4 Hz, H-1'''), 3.80 - 3.27 (m), 1.12 (1H, d, *J* = 6.4 Hz, H-6'''); ^{13}C NMR (100 MHz, CD_3OD): δ 178.1, 164.7, 161.6, 158.1, 157.2, 148.5, 144.5, 134.2, 122.2, 121.8, 116.4, 114.8, 104.3, 103.3, 101.1, 98.7, 93.6, 76.8, 75.8, 74.4, 72.6, 70.9, 70.7, 70.0, 68.4, 67.1, 16.6.

*Hexadecanoic acid methyl ester (**7**)*

Yellow oil, ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.66 (s, 3H, OCH_3), 2.29 (t, *J* = 13.8 Hz, H-14), 1.61 (1H, m, H-15), 1.27 (m, H-3), 0.88 (1H, t, *J* = 7.1 Hz, H-16); ^{13}C NMR (100 MHz, $CDCl_3$): δ 174.3, 51.4, 34.1, 32.0, 29.8, 29.7, 29.5, 29.4, 29.3, 29.2, 25.0, 22.8, 14.1.

*12-Hexadecenoic acid methyl ester (**8**)*

Yellow oil, ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.35 (m), 3.67 (s, OCH_3), 2.29 (t, *J* = 7.8 Hz), 2.06 – 2.00 (m), 1.68 – 1.59 (m), 1.3 (m), 0.88 (m); ^{13}C NMR (100 MHz, $CDCl_3$): δ 174.3, 130.0, 129.8, 51.4, 34.2, 32.0, 29.8, 29.7, 29.5, 29.4, 29.3, 29.2, 25.0, 22.8, 14.2.

*Nicotiflorin (**9**)*

Yellow powder, HRESITOFMS *m/z* 593.1489 $[M-H]^-$ (calcd. for $C_{27}H_{29}O_{15}$, 593.1479). ^{1}H NMR (400 MHz, CD_3OD): δ 8.11 (2H, d, *J* = 8.9 Hz, H-2' and H-6'), 6.93 (2H, *J* = 8.9 Hz, H-3' and H-5'), 6.45 (1H, d, *J* = 2.1 Hz, H-8), 6.25 (1H, d, *J* = 2.1 Hz, H-6), 5.11 (1H, d, *J* = 7.4 Hz, H-1''), 4.56 (1H, d, *J* = 1.4 Hz, H-1'''), 3.67 (1H, dd, *J* = 3.4 Hz and 1.6 Hz, H-2'''), 3.56 (1H, dd, *J* = 9.5 Hz and 3.5 Hz, H-3'''), 3.52 - 3.41 (8H, m), 1.16 (1H, d, J = 6.2 Hz, H-6'''); ^{13}C NMR (100 MHz,

CD_3OD): δ 179.4, 166.1, 161.5, 163.0, 159.4, 158.6, 135.5, 132.4, 122.8, 116.1, 105.6, 104.6, 102.4, 100.1, 95.0, 78.2, 77.2, 75.8, 73.9, 72.3, 72.1, 71.4, 68.6, 67.7, 17.9.

2.2.4. Hepatoprotective activity

HepG2 cells were kindly provided from Division of Antioxidant Research (Life Science Research Center, Gifu University). Cells were cultured in DMEM media (Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotics, penicillin-streptomycin (Gibco®, Life Technologies, Thermo Fisher Scientific Inc., MA and USA) and were maintained at 37ºC under a humidified atmosphere of 5% CO_2. HepG2 cells (2×10^4 cells/mL, 100 μL) were seeded in 96-well plates. After 24-h incubation, cells were pretreated with samples for 1 h, and were subsequently exposed to *t*-BHP (final concentration: 300 μM) for 3 h. A 10 μL amount of CCK-8 solution was added into the culture, and the plates were incubated for an additional 3 h. Visible absorption (490 nm) was measured using a microplate reader (E_{max} precision microplate reader, Molecular Devices Japan, Tokyo, Japan).

2.2.5. Antioxidant activity

Protocol of DPPH radical scavenging assay was performed essentially as described previously (Kato et al, 2016). A 10 μL amount of sample solutions and 190 μL of DPPH solution (78 μM in distilled H_2O/MeOH = 5/3) were added to 96-well plates, resulting in a final concentration of 74 μM for DPPH. The

solutions were vigorously mixed and allowed to stand. Visible absorption (λ = 545 nm) was measured after 30 min using a microplate reader (E_{max} precision microplate reader, Molecular Devices Japan, Tokyo, Japan). Wells without the compounds were considered as negative controls. At least three replicates were performed for each compound and control.

2.2.6. UPLC-ESITOFMS procedures

The samples were dissolved in DMSO/H_2O (1/1) at 20 mg/mL and filtered through 0.45 μm membrane filter (ADVANTEC®, Japan) and an aliquot (5 μL) of the sample was injected in the UPLC. Analysis was carried out by the Waters UPLC system (Aquity UPLC XevoQTof), using a UPLC BEH C_{18} analytical column (1.7 μm, 2.1 × 100 mm). The mobile phase contained solvent A (1% v/v AcOH in distilled water) and solvent B (acetonitrile). The liner gradient system employed was: 0 - 30 min 90% solvent A to 70% solvent A and 10% solvent B to 30% solvent B; kept for 5 min; 35 - 45 min 70% solvent A to 50% solvent A and 30% solvent B to 50% solvent B. The column eluate was monitored at 260 nm UV absorbance. Negative mode was employed in ESITOFMS.

2.3. Results and Discussion

2.3.1. Isolation of chemical constituents from Cinnamomum parthenoxylon leaves

The MeOH extract (214.6 g) of *C. parthenoxylon* leaves was partitioned successively with *n*-hexane and EtOAc to yield *n*-hexane fraction (25.4 g) and EtOAc fraction (39.8 g). The EtOAc fraction demonstrated the higher radical scavenging activity (**Figure 17**) and showed high inhibition on *t*-BHP-induced

cytotoxicity in HepG2 cells (**Figure 18**). Therefore, we carried out isolation of compounds responsible for the activities from the EtOAc fraction.

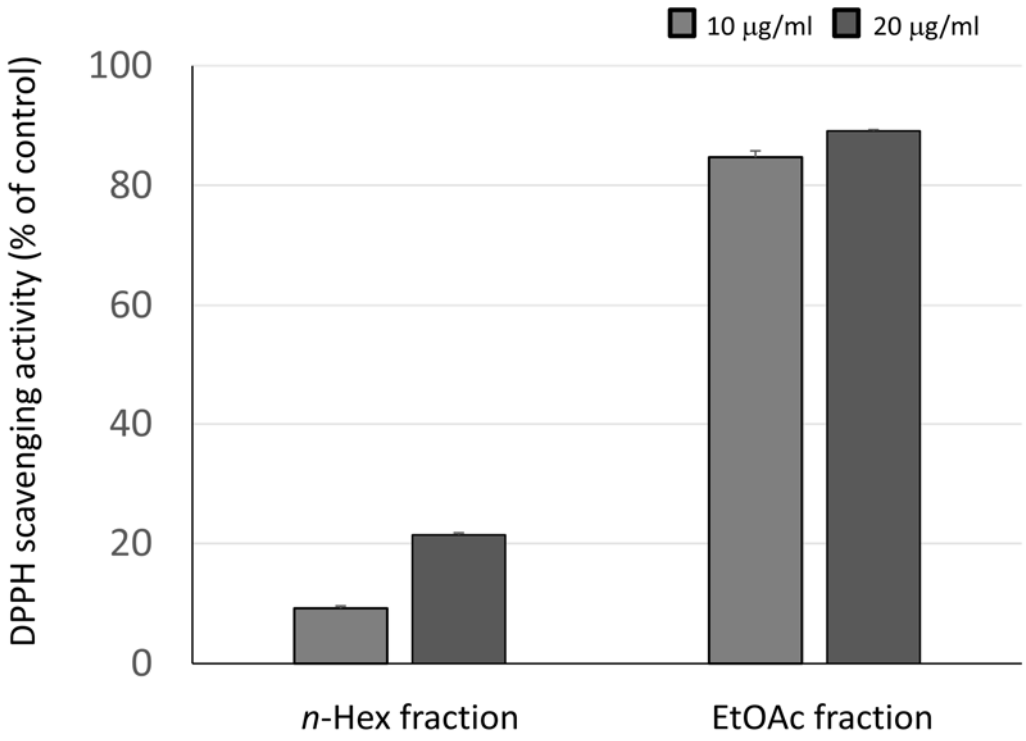


Figure 17. DPPH scavenging activity of *n*-Hex and EtOAc fractions of *Cinnamomum parthenoxylon* leaves (Means ± SEMs, *n* = 3).

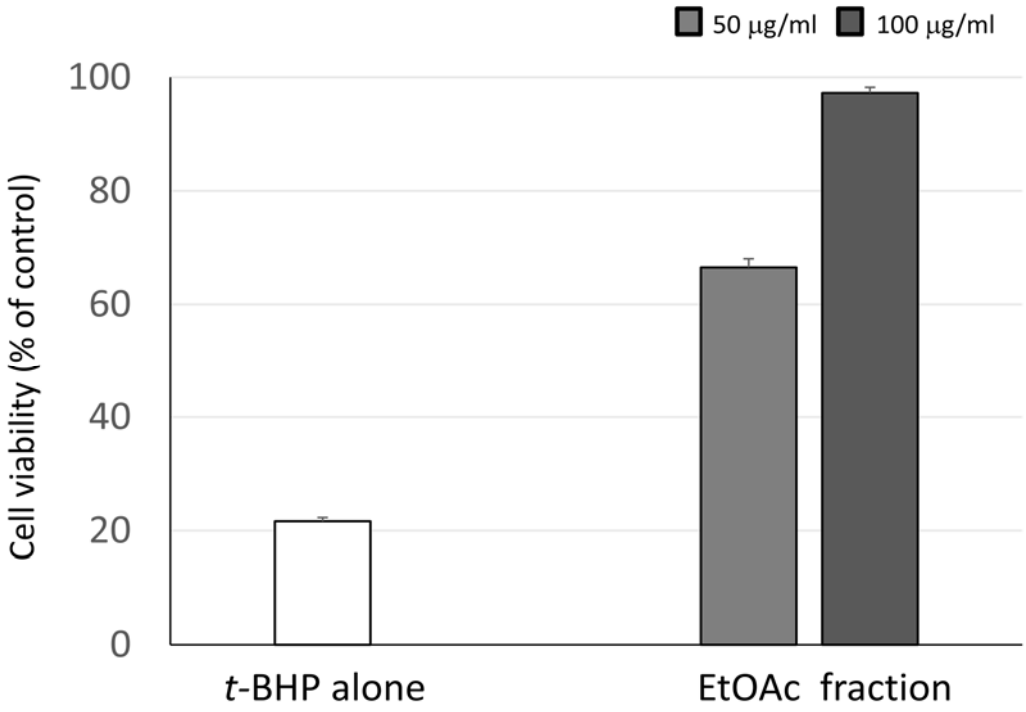


Figure 18. Hepatoprotective effect of EtOAc fraction of *Cinnamomum parthenoxylon* leaves on *t*-BHP induced cytotoxicity in HepG2 cells (Means ± SEMs, n = 3). Cells were treated with EtOAc fraction for 1 h, and then *t*-BHP was added at a final concentration of 300 µM and incubated for 3 h.

The fractions were separated by column chromatography (CC) on silica gel (SiO_2) and purified using Sephadex LH-20 CC and PTLC, to yield nine compounds. The chemical structures of the isolated compounds were elucidated by their ^{1}H, ^{13}C and 2D NMR spectra of each compound and literature data.

The HRESITOFMS peak at *m/z* 191.0328 $[M-H]^-$ (calcd. for $C_{10}H_7O_4$, 191.0344) suggested $C_{10}H_8O_4$ as the molecular formula of compound **1**. Its ^{1}H NMR displayed two doublet signals at δ_H 6.18 (1H, d, J = 9.6 Hz, H-3) and 7.84 (1H, d, J = 9.6 Hz, H-4), which are characteristic signals of a pyrone ring of the coumarin framework. Furthermore, aromatic singlet signals were observed at δ_H

6.79 (1H, s, H-8) and 7.19 (1H, s, H-5) and one methoxy group appeared at δ_H 3.90. These data determined the chemical structure of **1** as scopoletin (**Figure 19** and **Table 4**).

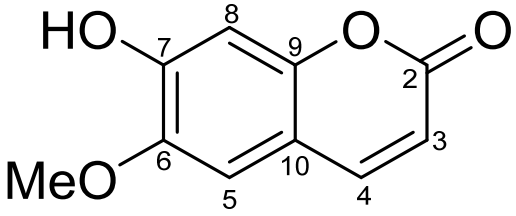


Figure 19. Chemical structure of scopoletin (**1**).

Table 4. ^{1}H and ^{13}C NMR data of compound **1**.

No	Compound **1** ($CDCl_3$)		Scopoletin ($CDCl_3$)*	
	δ_C	δ_H	δ_C	δ_H
2	160.5		162.8	
3	112.5	6.18, d, *J* = 9.6 Hz	112.2	6.18, d, *J* = 9.6 Hz
4	143.8	7.84, d, *J* = 9.6 Hz	144.5	7.87, d, *J* = 9.6 Hz
5	109.1	7.19, s	108.6	7.18, s
6	145.1		145.6	
7	151.0		151.3	
8	102.9	6.79, s	103.4	6.73, s
9	150.3		150.3	
10	111.3		111.4	
O-Me	55.9	3.90, s	56.4	3.80, s

*Razdan et al., 1987. *Phytochemistry 26*:2063-2069.

Compound **2** was isolated as a yellow powder and its molecular formula was established as $C_{27}H_{30}O_{14}$ from HRESITOFMS *m/z* 577.1555 $[M-H]^-$ (calcd. for

$C_{27}H_{29}O_{14}$, 577.1557). Two set of two doublets observed in the aromatic region at δ_H 7.95 and 6.95, and at δ_H 6.88 and 6.80, and a carbonyl carbon observed at δ_C 182.5 are characteristic of an apigenin skeleton. The presence of a rutinoside moiety was confirmed by proton signals at δ_H 5.24 - 1.21 and respective carbons. The HMBC clearly indicated that a β-glucoside unit in the rutinoside structure was attached to the 7-OH of an apigenin skeleton, resulting in the structural determination of **2** as isorhoifolin. The key ^{1}H - ^{1}H COSY and HMBC correlation of compound **2** is shown in **Figure 20** and the NMR spectra data of compound **2** comparing with literature data was given in **Table 5**.

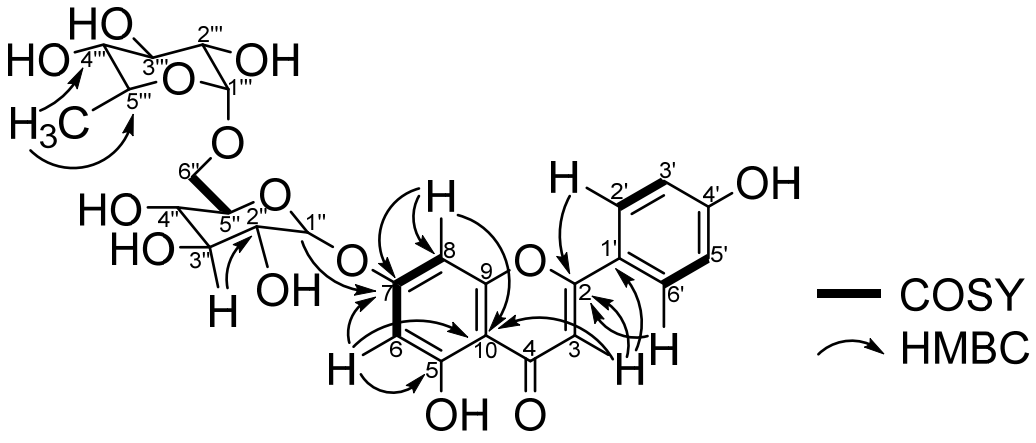


Figure 20. Key ^{1}H - ^{1}H COSY and HMBC correlation of compound **2**.

Table 5. ^{1}H and ^{13}C NMR data of compound **2**.

No	Compound **2** (DMSO-d_6)		Isorhoifolin (DMSO-d_6)*	
	δ_C	δ_H	δ_C	δ_H
2	164.8		164.8	
3	103.7	6.88, s	103.2	6.78, s
4	182.5		182.1	
5	161.6		161.6	
6	98.3	6.38, d, *J* = 1.8 Hz	99.3	6.18, d, *J* = 1.98 Hz
7	163.1		164.1	
8	95.0	6.80, d, *J* = 2.3 Hz	94.4	6.47, d, *J* = 1.98 Hz
9	157.5		157.8	
10	106.0		104.0	
1'	121.1		121.6	
2'	129.1	7.95, d, *J* = 9.2 Hz	128.9	7.91, d, *J* = 8.76 Hz
3'	116.6	6.95, d, *J* = 9.2 Hz	116.4	6.93, d, *J* = 8.76 Hz
4'	161.9		161.9	
5'	116.6	6.95, d, *J* = 9.2 Hz	116.4	6.93, d, *J* = 8.76 Hz
6'	129.1	7.95, d, *J* = 9.2 Hz	128.9	7.91, d, *J* = 8.76 Hz
1"	99.8	5.24, d, *J* = 7.3 Hz	100.8	5.04, d, *J* = 7.08 Hz
2"	71.0	3.77 – 3.21, m	72.4	3.90 – 3.10, m
3"	76.8	3.77 – 3.21, m	75.9	3.90 – 3.10, m
4"	70.1	3.77 – 3.21, m	70.1	3.90 – 3.10, m
5"	77.5	3.77 – 3.21, m	76.8	3.90 – 3.10, m
6"	61.0	3.77 – 3.21, m	66.3	3.90 – 3.10, m
1'"	101.0	5.14, brs	101.3	4.54, brs
2'"	70.9	3.77 – 3.21, m	71.4	3.90 – 3.10, m
3'"	70.6	3.77 – 3.21, m	70.7	3.90 – 3.10, m
4'"	72.4	3.77 – 3.21, m	73.4	3.90 – 3.10, m
5'"	68.9	3.77 – 3.21, m	68.7	3.90 – 3.10, m
6'"	18.6	1.21, d, *J* = 6.4 Hz	18.3	1.10, d, *J* = 6.41 Hz

*Aksit et al., 2014. *Rec. Nat. Prod. 8*: 277-280.

Compounds **6** and **9** also showed the flavonol rutinoside characteristics and were confirmed as rutin and nicotiflorin, respectively, (Ganbaatar et al., 2015) (**Figure 21** and **Table 6**).

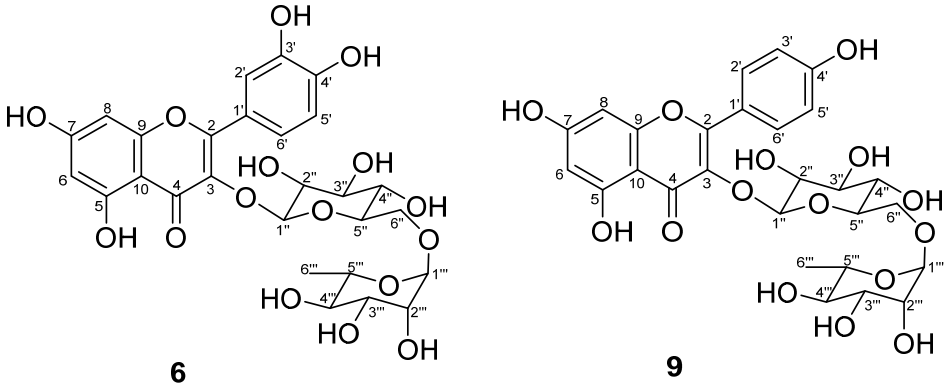


Figure 21. Chemical structure of rutin (**6**) and nicotiflorin (**9**).

Table 6. ^{1}H and ^{13}C NMR data of rutin (**6**) and nicotiflorin **(9).**

No	Rutin (**6**) (CD_3OD)		Nicotiflorin **(9)** (CD_3OD)	
	δ_C	δ_H	δ_C	δ_H
2	158.1		159.4	
3	134.2		135.5	
4	178.1		179.4	
5	161.6		163.0	
6	98.7	6.21, d, *J* = 2.3 Hz	100.1	6.25, d, *J* = 2.1 Hz
7	164.7		166.1	
8	93.6	6.40, d, *J* = 2.3 Hz	95.0	6.45, d, *J* = 2.1 Hz
9	157.2		158.6	
10	104.3		105.6	
1'	121.8		122.8	
2'	116.4	7.67, d, *J* = 2.3 Hz	132.4	8.11, d, *J* = 8.9 Hz
3'	144.5		116.1	6.93, d, *J* = 8.9 Hz
4'	148.5		161.5	
5'	114.8	6.87, d, *J* = 8.7 Hz	116.1	6.93, d, *J* = 8.9 Hz
6'	122.2	7.62, dd, *J* = 8.0, 2.3 Hz	132.4	8.11, d, *J* = 8.9 Hz
1"	103.3	5.11, d, *J* = 7.8 Hz	104.6	5.11, d, *J* = 7.4 Hz
2"	74.4	3.80 – 3.27, m	75.8	3.52 – 3.28, m
3"	76.8	3.58, dd, *J* = 9.5, 3.5 Hz	78.2	3.52 – 3.28, m
4"	70.7	3.80 – 3.27, m	71.4	3.52 – 3.28, m
5"	75.8	3.80 – 3.27, m	77.2	3.52 – 3.28, m
6"	67.1	3.80 – 3.27, m	67.7	3.52 – 3.28, m
1'"	101.1	4.52, d, *J* = 1.4 Hz	102.4	4.56, d, *J* = 1.4 Hz
2'"	70.0	3.63, dd, *J* = 3.4, 1.8 Hz	72.1	3.67, dd, *J* = 3.4, 1.6 Hz
3'"	70.9	3.80 – 3.27, m	72.3	3.52 – 3.28, m
4'"	72.6	3.80 – 3.27, m	73.9	3.52 – 3.28, m
5"	68.3	3.80 – 3.27, m	68.6	3.52 – 3.28, m
6'"	16.5	1.12, d, *J* = 6.4 Hz	17.9	1.16, d, *J* = 6.2 Hz

Compound **3** is yellow powder, its molecular formula was established as $C_{15}H_{14}O_6$ from HRESITOFMS *m/z* 289.0712 $[M-H]^-$ (calcd. for $C_{15}H_{13}O_6$, 289.0714). ^1H NMR spectrum of **3** showed the presence of two *meta*-coupled doublet proton on the A ring at δ_H 5.92 (1H, d, *J* = 2.3 Hz, δ_C 94.9), 6.03 (1H, d, *J* = 2.3 Hz, δ_C 95.3), which were assigned to H-8 and H-6, respectively. The remaining aromatic protons at δ_H 7.06 (1H, d, *J* = 1.8 Hz), 6.79 (1H, d, *J* = 8.2 Hz) and 6.84 (1H, dd, *J* = 8.5 and 1.8 Hz) were assigned to H-2', H-5' and H-6', respectively. The ^{13}C NMR spectrum displayed 14 carbon signals (**Figure 22** and **Table 7**). The HRESITOFMS, ^1H and ^{13}C NMR data confirmed that **3** were epicatechin (Davis et al., 1996).

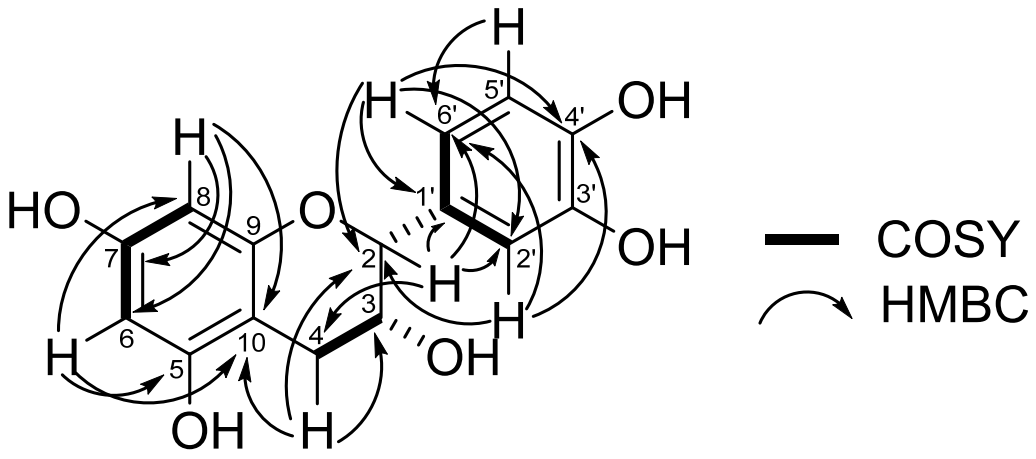


Figure 22. Key ^1H - ^1H COSY and HMBC correlation of compound **3**.

Table 7. ^{1}H and ^{13}C NMR data of compound **3**.

No	Compound **3** (aceton-d_6)		Epicatechin (aceton-d_6)*	
	δ_C	δ_H	δ_C	δ_H
2	78.6	4.88, s	79.5	4.88, s
3	66.1	4.21, s	67.0	4.21, s
4α	28.8	2.74, dd, *J* = 16.7, 3.2 Hz	29.0	2.74, dd, *J* = 16.5, 3.2 Hz
4β		2.87, dd, *J* = 16.7, 4.6 Hz		2.87, dd, *J* = 16.5, 4.6 Hz
5	156.8		157.6	
6	95.3	6.03, d, *J* = 2.3 Hz	96.2	6.02, d, *J* = 2.0 Hz
7	156.7		157.6	
8	94.9	5.92, d, *J* = 2.3 Hz	95.8	5.92, d, *J* = 2.0 Hz
9	156.3		157.2	
10	99.0		99.9	
1'	131.4		132.3	
2'	114.5	7.06, d, *J* = 1.8 Hz	115.3	7.05, d, *J* = 1.4 Hz
3'	144.6		145.4	
4'	144.5		145.3	
5'	114.7	6.79, d, *J* = 8.2 Hz	115.5	6.78, d, *J* = 8.1 Hz
6'	118.5	6.84, dd, *J* = 8.5, 1. 8 Hz	119.4	6.84, dd, *J* = 8.2, 2.0 Hz

*Davis et al., 1996. *Magn. Reson. Chem. 34*:887-890.

Compound **4** was a pale yellow powder and its molecular formula was indicated as $C_{13}H_{22}O_3$ from HRESITOFMS peak at *m/z* 225.1479 $[M-H]^-$ (calcd. for $C_{13}H_{21}O_3$, 225.1491).The spectral data of compound **4** were identical to those previously reported (Marino et al., 2004), **4** was confirmed as blumenol A (**Figure 23** and **Table 8**).

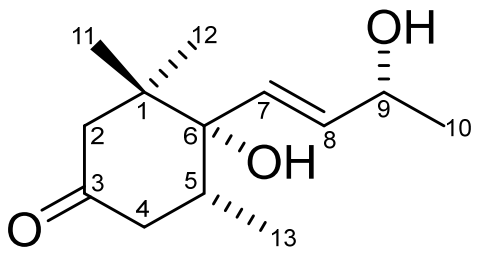


Figure 23. Chemical structure of blumenol A (**4**).

Table 8. ^{1}H and ^{13}C NMR data of compound **4**.

	Compound **4** ($CDCl_3$)		Blumenol A ($CDCl_3$)*	
No	δ_C	δ_H	δ_C	δ_H
1	42.6		43.8	
2 ax		2.84, d, *J* = 13.3 Hz		2.87, d, *J* = 13.4
2 eq	51.5	1.92, dd, *J* = 13.8, 2.3 Hz	52.2	1.82, dd, *J* = 13.4, 2.0 Hz
3	211.3		214.6	
4 ax		2.40, d, *J* = 12.8 Hz		2.45, d, *J* = 13.4
4 eq	45.2	2.13, dd, *J* =13.4, 2.1 Hz	45.9	2.13, dd, *J* = 13.4, 2.1 Hz
5	36.4	2.29, m	37.5	2.27, m
6	77.3		78.0	
7	131.9	5.70, d, *J* = 16.9 Hz	133.8	5.66, d, *J* = 15.8 Hz
8	135.2	5.83, dd, *J* = 15.8, 5.5 Hz	135.3	5.83, dd, *J* = 15.8, 5.9 Hz
9	68.4	4.44, q,	69.4	4.34, q
10	23.9	1.33, d, *J* = 6.4 Hz	24.2	1.27, d, *J* = 6.4 Hz
11	24.5	0.97, s	25.9	0.98, s
12	24.4	0.94, s	25.2	0.92, s
13	15.9	0.90 d, *J* = 6.6 Hz	16.3	0.90, d, *J* = 6.6 Hz

*Marino et al., 2004. *J. Agric. Food Chem. 52*:7525-7531.

The ^{1}H NMR spectrum of compound **5** exhibited two doublet peaks at δ_H 7.92 (2H, d, *J* = 9.2 Hz) and 6.91 (2H, d, *J* = 9.2 Hz) equivalent to four protons (H-2 and H-6) and (H-3 and H-5) due to the presence of disubstituted benzene ring. The ^{13}C NMR showed five peaks assigned for the carbon of carboxylic acid and benzene ring, compound **5** identified as 4-hydroxybenzoic acid (**Figure 24** and **Table 9**) (Yoshioka et al., 2004).

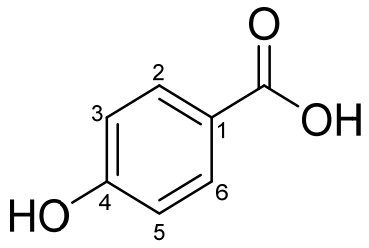
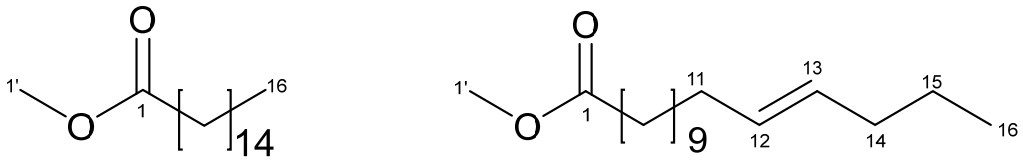


Figure 24. Chemical structure of 4-hydroxybenzoic acid (**5**).

Table 9. ^{1}H and ^{13}C NMR of 4-hydroxybenzoic acid (**5**).

No	4-hydroxybenzoic acid (**5**)	
	δ_C	δ_H
2	132.7	7.92, d, *J* = 9. 2 Hz
3	115.9	6.91, d, *J* = 9.2 Hz
4	162.5	
5	115.9	6.91, d, *J* = 9.2 Hz
6	132.7	7.92, d, *J* = 9. 2 Hz
C=O	167.8	

Compounds **7** and **8** were obtained as yellow oil. In ^{13}C NMR spectra, 16 carbon signals were observed, including the carbonyl carbon signal at δ_C 174.3 and methoxy group at δ_C 51.7, which was suggestive of a carboxylic acid ester, compound **7** identified as hexadecanoic acid methyl ester. Furthermore, presence of carbon signals at δ_C 130.0 and 129.8, identified compound **8** as 12-hexadecenoic acid methyl ester (**Figure 25** and **Table 10**) (Ajoku et al., 2015).



7 **8**

Figure 25. Hexadecanoic acid methyl ester (**7**) and 12-hexadecenoic acid methyl ester (**8**).

Table 10. ^{13}C NMR of compound **7** and compound **8**.

No	Hexadecanoic acid methyl ester ($CDCl_3$)*	Compound **7** ($CDCl_3$)	Compound **8** ($CDCl_3$)
	δ_C	δ_C	δ_C
1	174.6	174.3	174.3
2	22.5	22.8	22.8
3	29.2	25.0	25.0
4	29.3	29.2	29.2
5	29.4	29.3	29.3
6	29.4	29.3	29.3
7	29.5	29.4	29.4
8	29.5	29.4	29.5
9	29.5	29.4	29.5
10	29.6	29.5	29.7
11	29.6	29.5	29.8
12	29.6	29.7	129.8
13	29.7	29.8	130.0
14	31.8	32.0	32.0
15	33.9	34.1	34.2
16	14.0	14.1	14.2
1'	51.7	51.4	51.4

*Ajoku et al., 2015. *Nat. Prod. Chem. Res. 3*:169-174.

2.3.2. UPLC-ESITOFMS analysis

Analysis of the chemical constituent from EtOAC fraction of *C. parthenoxylon* leaves were carried out using UPLC-ESITOFMS (Pardede and

Koketsu, 2017). The chemical constituent was assigned as $[M-H]^-$ ions. The analysis revealed three major constituents in the EtOAc fraction of *C. parthenoxylon* leaves, this confirmed rutin (**6**, retention time: 12.19 min), nicotiflorin (**9**, retention time: 15.22 min) and isorhoifolin (**2**, retention time: 17.44 min) in UPLC-ESITOFMS chromatogram, respectively. The retention time at 15.22 min was identified as nicotiflorin (**9**) in comparison with the retention time and the molecular weight of nicotiflorin (**9**) (Farias and Mendez, 2014). Flavonoid rutinosides, rutin (**6**), nicotiflorin (**9**) and isorhoifolin (**2**) showed to be major constituents in the EtOAc fraction of *C. parthenoxylon* leaves based on UPLC-ESITOFMS analysis (**Figure 26**). The HRESITOFMS spectrum of each retention time of compounds isolated from *Cinnamomum parthenoxylon* leaves were given in **Figure 27**.

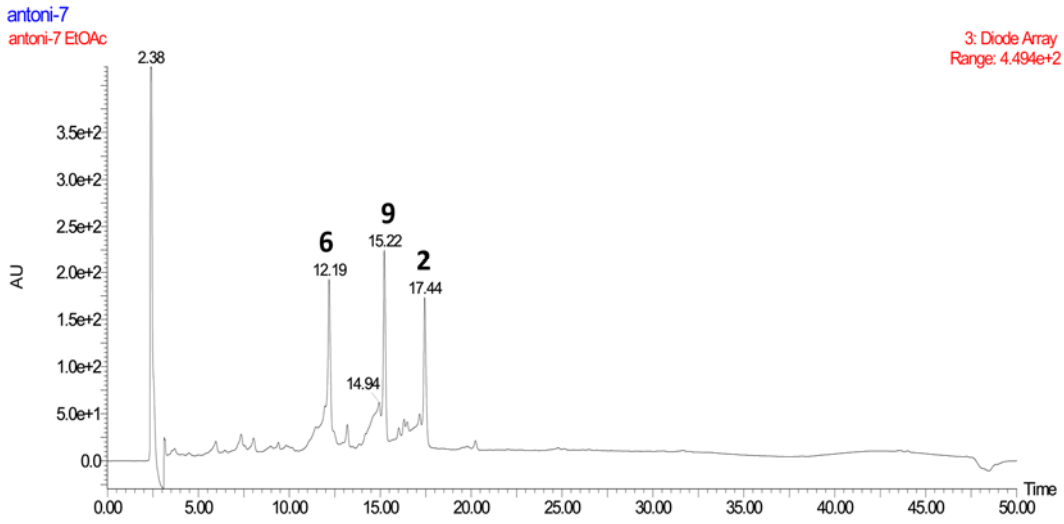


Figure 26. UPLC-ESITOFMS chromatogram of the EtOAc fraction from *Cinnamomum parthenoxylon* leaves. Rutin (**6**), nicotiflorin (**9**) and isorhoifolin (**2**).

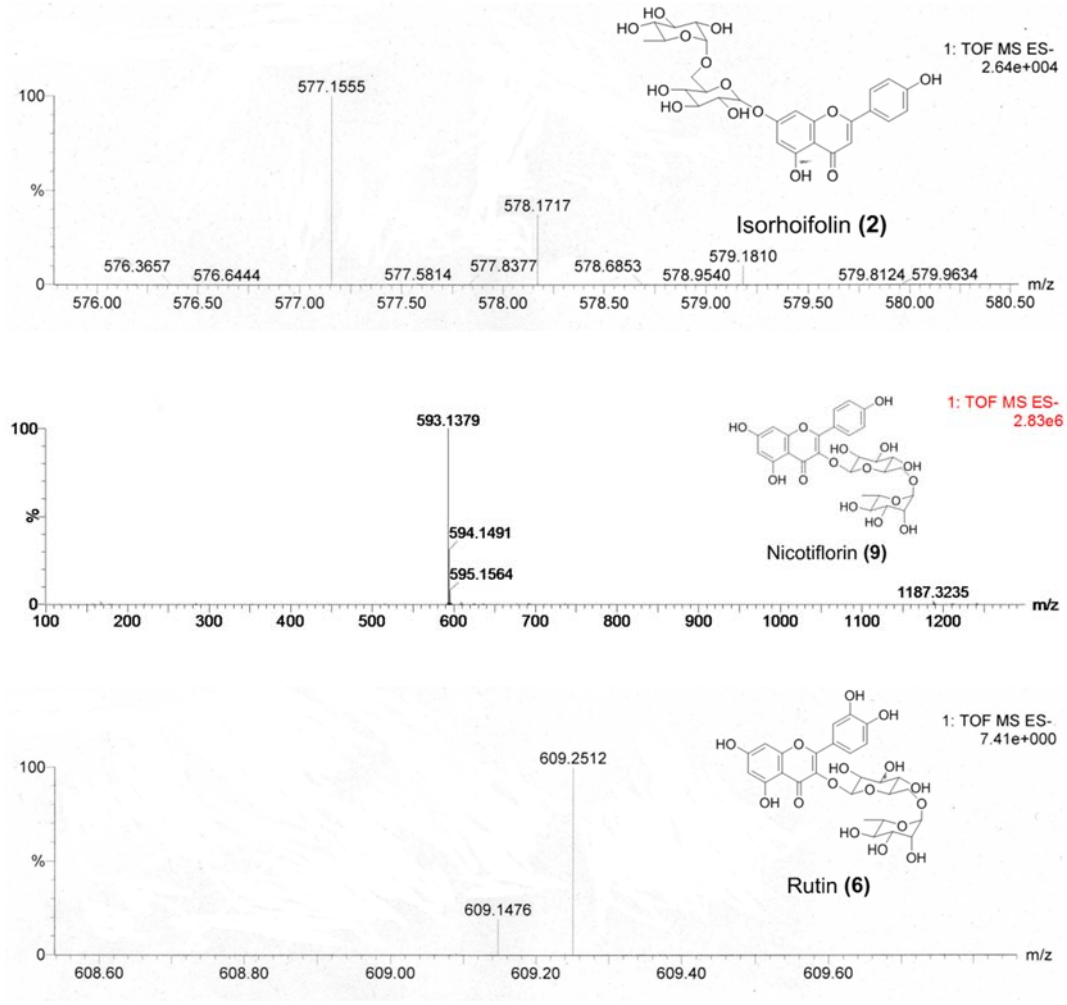


Figure 27. The HRESITOFMS spectrum of each retention time of compounds isolated from *Cinnamomum parthenoxylon* leaves.

2.3.3. Hepatoprotective and antioxidant activity

Hepatoprotective and antioxidant activity of the obtained flavonoid rutinosides was evaluated. The hydroxy group is important for free radical scavenging efficiency, in particular, the B ring hydroxy group of flavonoids has potential for antioxidant activity (Rusak et al., 2005). Rutin (**6**) showed higher antioxidant activity compared to nicotiflorin (**9**) and isorhoifolin (**2**) in both

concentrations of 10 μM and 20 μM, respectively. The major difference in the structure between rutin (**6**) and nicotiflorin (**9**) is the absence of hydroxy group at the C-3' position in the structure of nicotiflorin (**9**). The hydroxy group at the C-3' position is essential for the antioxidant activity (Loganayaki et al., 2013). On the other hand, the presence of rutinoside group at C-7 and the absence of hydroxy group at C-3' in isorhoifolin (**2**) results in extremely low antioxidant activity (**Figure 28**).

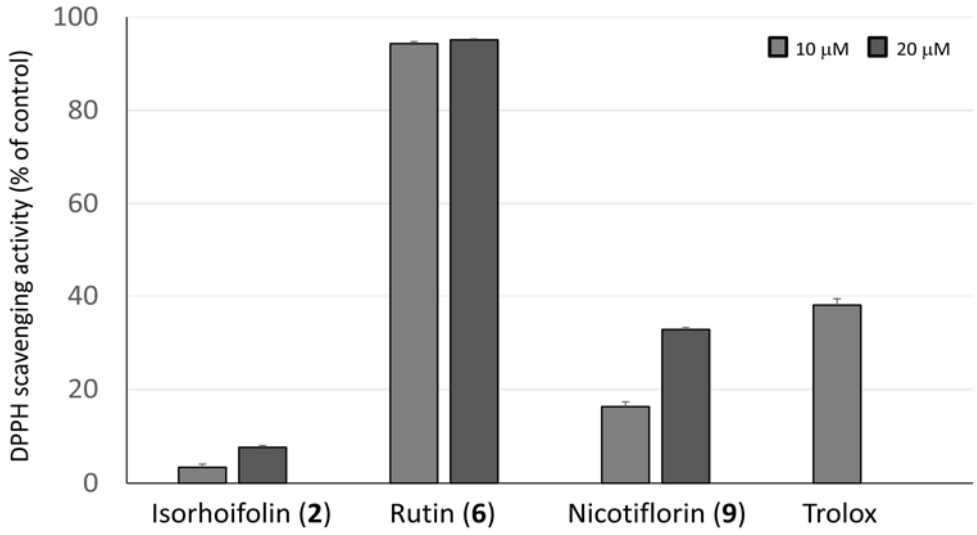


Figure 28. DPPH scavenging activity of isolated flavonoid rutinosides (Means ± SEMs, *n* = 3). Trolox (10 μM) was used as a positive control.

Tert-buthyl hydroperoxide (*t*-BHP) is a well-known toxic agent that can induce oxidative stress that has been recognized to be a significant factor in several diseases including liver diseases (Jung et al., 2015). Research for hepatoprotective effects of active compounds on *t*-BHP-induced HepG2 was conducted. The hepatoprotective activity of rutin (**6**), nicotiflorin (**9**) and

isorhoifolin (**2**) was evaluated in two concentrations of 50 µM and 100 µM, respectively. The dose-dependent effect of each compound confirmed that rutin (**6**) has the highest hepatoprotective activity as compared with nicotiflorin (**9**) and isorhoifolin (**2**). The absence of hydroxy group at the C-3' position in B ring of nicotiflorin (**9**) and isorhoifolin (**2**), and additional rutinoside group at C-7 of A ring in the structure of isorhoifolin (**2**) decreased their hepatoprotective activity (**Figure 29**).

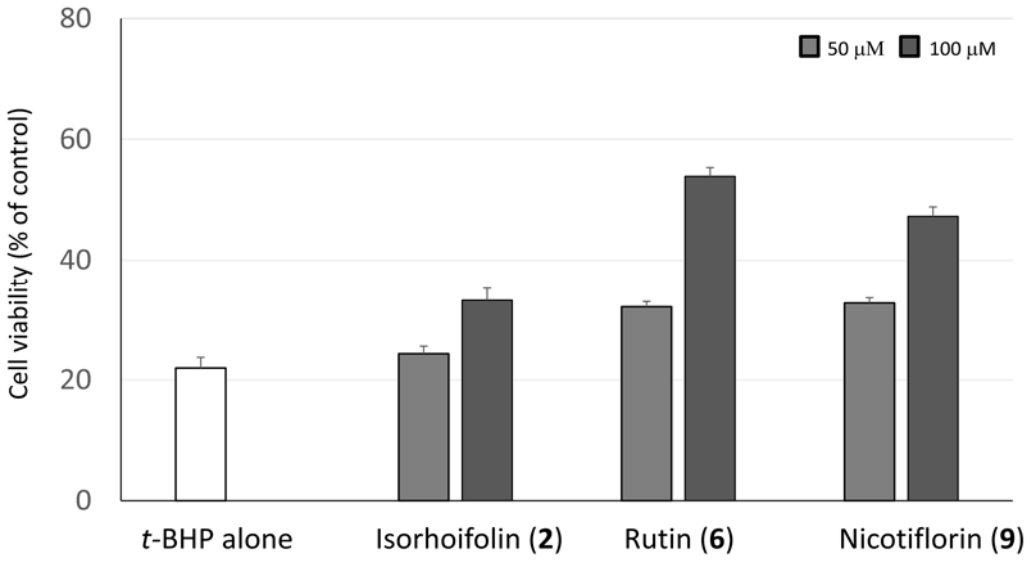


Figure 29. Hepatoprotective effect of EtOAc fraction of isolated flavonoid rutinosides on *t*-BHP induced cytotoxicity in HepG2 cells (Means ± SEMs, $n = 3$).

Quercetin and kaempferol showed stronger activity on HepG2 cell cytotoxicity (Kinjo et al., 2006), compared with those, rutin (**6**) and nicotiflorin (**9**) were weaker. The difference between the structures of rutin (**6**) and nicotiflorin (**9**) as compared with quercetin and kaemferol, respectively, is the

presence of the rutinosides moiety at C3 carbon. Introduction of rutinosides moiety into quercetin and kaemferol might reduce the activity. Interestingly, even if the skeleton of compounds was the same, but the patterns of functional groups were highly influential on activities.

2.4. Conclusions

In conclusion, we successfully confirmed that the catechol group at B ring existed in rutin (**6**) is potential for hepatoprotective and antioxidant activity among the investigated flavonoid rutinosides. Our findings provide more scientific evidence for the biological activity of *C. parthenoxylon* leaves and the isolated flavonoid rutinosides are partly responsible for the ability.

References

Adfa, M., Rahmat, R., Ninomiya, M., Yudha, S., Tanaka, K., Koketsu, M. 2016. Antileukemic activity of lignans and phenylpropanoids of *Cinnamomum parthenoxylon*. *Bioorg. Med. Chem. Lett. 26*:761-764.

Ajoku, G. A., Okwute, S. K., Okogun, J. I. 2015. Isolation of Hexadecanoic Acid Methyl Ester and 1,1,2-Ethanetricarboxylic Acid-1-Hydroxy-1, 1-Dimethyl Ester from the Calyx of Green *Hibiscus Sabdariffa* (Linn). *Nat. Prod. Chem. Res. 3*:169-174.

Aksit, H., Celik, S. M., Sen, O., Erenler, R., Demirtas, I., Telci, I., Elmastas, M. 2014. Complete isolation characterization of polar portion of *Menta dumetorum* water extract. *Rec. Nat. Prod. 8*:277-280.

Cao, H., Graves, D. J., Anderson, R. A. 2010. Cinnamon extract regulates glucose transporter and insulin-signaling gene expression in mouse adipocytes. *Phytomedicine 17*:1027-1032.

Daker, M., Lin, V. Y., Akowuah, G. A., Yam, M. F., Ahmad, M. 2013. Inhibitory effects of *Cinnamomum burmannii* Blume stem bark extract and trans-cinnamaldehyde on nasopharyngeal carcinoma cells; synergism with isplatin. *Exp. Ther. Med. 5*:1701-1706.

Davis, A. L., Cai, Y., Dvies, A. P., Lewis, J. R. 1996. 1H and 13C NMR assignment of some green tea polyphenols. *Magn. Reson. Chem. 34*:887-890.

Farias, L. D. S., Mendez, A. S. L. 2014. LC/ESI-MS method applied to characterization of flavonoids glycosides in *B. forficate* subsp pruinosa**.** *Quim Nova 37*:483-486.

Ganbaatar, C., Gruner, M.,Mishig, D., Duger, R., Schmidt, A. W., Knolker H. J. 2015. Flavonoid Glycosides from the Aerial Parts of *Polygonatum odoratum* (Mill.) Druce Growing in Mongolia. *Nat. Prod. J. 8*:1-7.

Hong, C. H., Hur, S. K., Oh, O. J., Kim, S. S., Nam, K. A., Lee, S. K. 2002. Evaluation of natural products on inhibition of inducible cyclooxygenase (COX-2) and nitric oxide synthase (iNOS) in cultured mouse macrophage cells. *J. Ethnopharmacol 8*:153-159.

Jia, Q., Liu, X., Wu, X., Wang, R., Hu, X., Li, Y., Huang, C. 2009. Hypoglycemic activity of a polyphenolic oligomer-rich extract of *Cinnamomum parthenoxylon* bark in normal and streptozotocin-induced diabetic rats. *Phytomedicine 16*:744-750.

Jung, J. W., Ko, W. M., Park, J. H., Seo, K. H., Oh, E. J., Lee, D. Y., Lee, D. S., Kim, Y. C., Lim, D. W., Han, D., Baek, N. I. 2015. Isoprenylated flavonoids

from the root bark of *Morus alba* and their hepatoprotective and neuroprotective activities. *Arch. Pharm. Res. 38*:2066-2075.

Kato, K., Ninomiya, M., Tanaka, K., Koketsu, M. 2016. Effects of functional groups and sugar composition of quercetin derivatives on their radical scavenging properties. *J. Nat. Prod. 79*:1808-1814.

Kawatra, P., Rajagopalan, R. 2015. Cinnamon: Mystic powers of a minute ingredient. *Pharmacognosy Res. 7*:1-6.

Kinjo, J., Hitoshi, M., Tsuchihashi, R., Korematsu, Y., Miyakoshi, M., Murakami, T., Niiho, D., Mizutani, K., Tanaka, T., Nonaka, G., Nohara, T., Okawa, M., Okabe, H. 2006. Hepatoprotective constituents in plants 15: protective effects of natural-occuring flavonoids and miscellaneous phenolic compounds as determined in an HepG2 cell cytotoxicity assay. *J Nat Med 60*:36-41.

Lee, M. A., Park, H. J., Chung, H. J., Kim, W. K., Lee, S. K. 2013. Antitumor activity of 2-hydroxycinnamaldehyde for human colon cancer cells through suppression of β-catenin signaling. *J. Nat. Prod. 76*:1278-1284.

Loganayaki, N., Siddhuraju, P., Manian, S. 2013. Antioxidant activity and free radical scavenging capacity of phenolic extracts from *Helicteres isora* L. and *Ceiba pentandra* L. *J. Food. Sci. Technol. 50*:687-695.

Marino, S. D., Borbone, N., Zollo, F., Tanaro, A., Meglio, P. D., Lorizzi, M. 2004. Megastigmane and phenolic components from *Laurus nobilis* L. and their inhibitory effects on Nitric oxide production. *J. Agri. Food. Chem. 52*:7525-7531.

Ninomiya, M., Nishida, K., Watanabe, K., Koketsu, M. 2013. Structure-activity relationship studies of 5,7-dihydroxyflavones as naturally occurring inhibitors of cell proliferation in human leukemia HL-60 cells. *J. Nat. Med. 67*:460-467.

Pardede, A., Koketsu, M. 2017. Antioxidant and antileukemic activity of chemical components from bark of *Mangifera casturi*. *Comp. Clin. Pathol. 26*:499-504.

Pardede, A., Mashita, K., Ninomiya, M., Tanaka, K., Koketsu, M. 2016. Flavonoid profile and antileukemic activity of *Coreopsis lanceolata* flowers. *Bioorg. Med. Chem. Lett. 26:*2784-2787.

Razdan, T. K., Qadri, B., Harkar, S., Waight, E. S. 1987. Chromones and coumarins from *Skimmia lauriola. Phytochemistry 26*:2063-2069.

Rusak, G., Gutzeit, H. O., Muller, J. L. 2005. Structurally related flavonoids with antioxidative properties differentially progression and apoptosis of human acute leukemia cells. *Nutrition Res. 25*:141-153.

Unlu, M., Ergene, E., Unlu, G. V., Zeytinoglu, H. S., Vural, N. 2010. Composition, antimicrobial activity and in vitro cytotoxicity of essential oil from *Cinnamomum zeylanicum* Blume (Lauraceae). *Food Chem. Toxicol. 48*:3274-3280.

Wang, Y. H., Avula, B., Nanayakkara, N. P., Zhao, J., Khan, I. A. 2013. *Cassia Cinnamon* as a Source of Coumarin in Cinnamon-Flavored Food and Food Supplements in the United States. *J. Agric. Food Chem. 61*:4470-4476.

Wei, X., Li, G. H., Wang, X. L., He, J. X., Wang, X. N., Ren, D. M., Lou, H. X., Shen, T. 2017. Chemicals constituents from the leaves of *Cinnamomum parthenoxylon* (Jack) Meisn. (Lauraceae). *Biochem. Sys. Ecol. 70*:95-98.

Yoshioka, T., Inokuchi, T., Fujioka, S., Kimura, Y. 2004. Phenolic compounds and flavonoids as plant growth regulators from fruit and leaf of *Vitex rotundifolia*. *Z Naturforsch 59*:509-514.

Chapter 3

Isolation of secondary metabolites from *Stenochlaena palustris* stems and structure activity relationship of 20-hydroxyecdysone derivatives on antitermite activity

3.1. Introduction

Termites are devastating insect pests that frequently cause huge damage to the living environment, particularly to buildings and agricultural crops. *Coptotermes curvignathus* Holmgren (Subterranean termite) is the major termite species and is widely distributed in Southeast Asia such as Indonesia and Malaysia. In these countries, *C. curvignathus* Holmgren is known to cause significant loss to the finished and unfinished wooden materials (Sowmya et al., 2016; Adfa et al., 2017). The soldier and worker of *Coptotermes curvignathus* Holmgren are show in **Figure 30**.

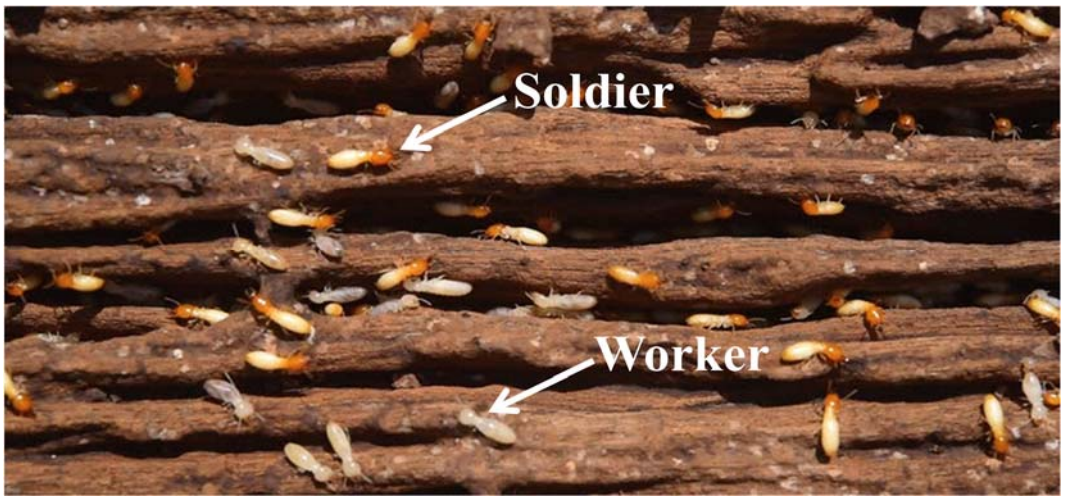


Figure 30. The soldier and worker of *Coptotermes curvignathus* Holmgren.

Plants play an important role as a source of innovative molecules for pest control. Exploration of natural alternatives from plants has attracted substantial attention worldwide. Indeed, certain data suggest that phytochemicals and essential oils showed antitermite activity against *C. curvignathus* Holmgren (Roszaini et al., 2013; Oramahi and Yoshimura, 2013; Adfa et al., 2015).

Figure 31. *Stenochlaena palustris*, tree (a), stem has been chopped (b), leaves (c) and young leaves sold on Indonesia local market.

Stenochlaena palustris belonging to the *Blechnaceae* family (**Figure 31**), is an edible fern which is called kelakai in South Kalimantan, Indonesia. Previous phytochemical investigations reported that chemical constituents isolated from *S.*

palustris exhibited potent biological activities for human health including antioxidant, antidiabetic, and cholinesterase inhibitory effects (Liu et al., 1998; Zuraini et al., 2010; Chai et al., 2015; Chear et al., 2016). Locally, *S. palustris* is known to be resistant to the attack of pathogenic organisms. Giron and San Pablo demonstrated that *S. palustris* displayed resistance to wood-decay fungi under varied laboratory conditions (Giron and San Pablo, 2004).

Our efforts have been directed to search for the possibilities of natural compounds as termite control agents. Within the scope of our ongoing program, we explored antitermite molecules from *S. palustris* stems. Furthermore, we examined the structure-activity relationships of 20-hydroxyecdysone derivatives on their antitermite properties against *C. curvignathus* Holmgren.

3.2. Materials and Methods

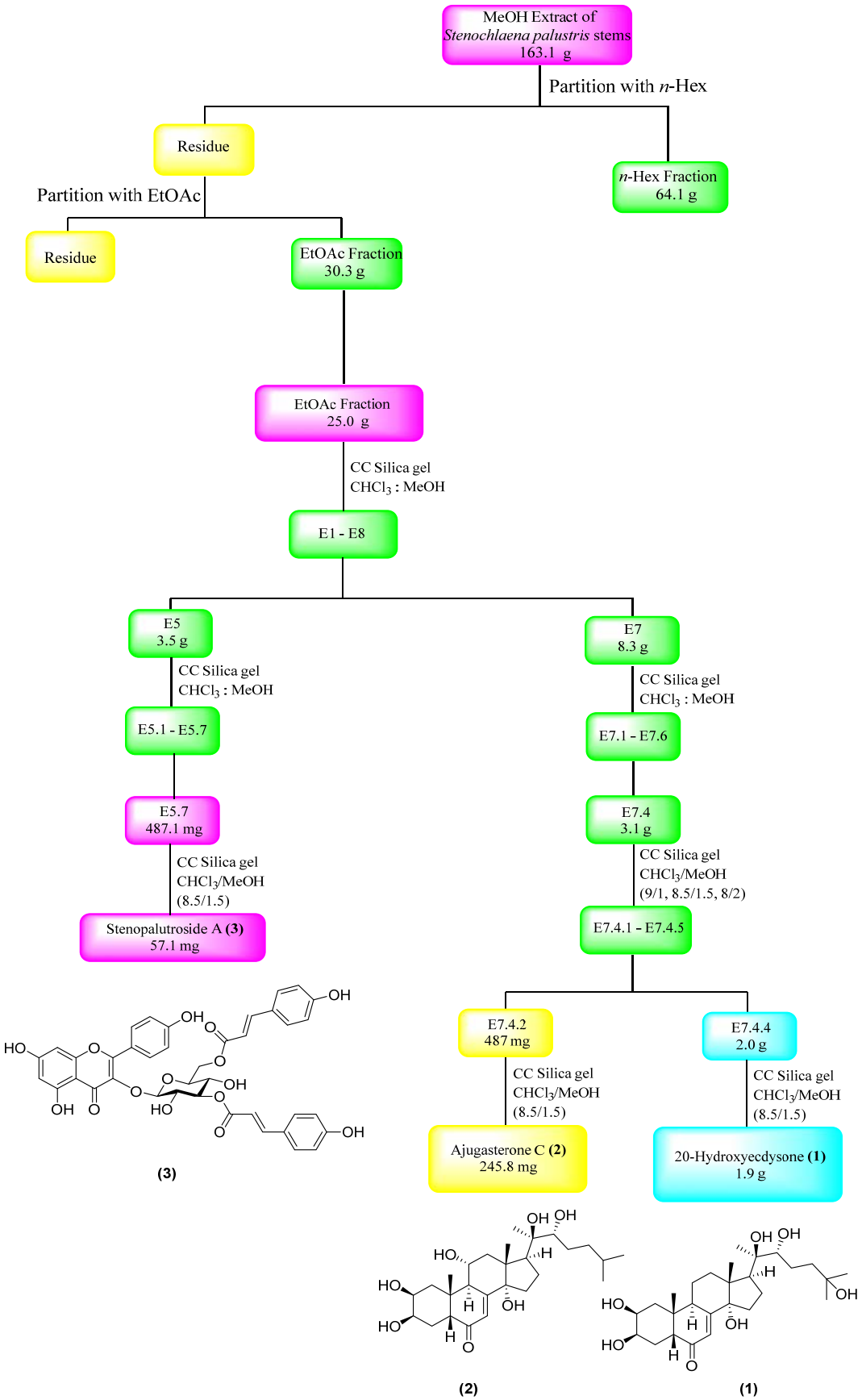
3.2.1. General experimental procedures

^{1}H and ^{13}C NMR spectra were recorded on a JEOL ECA 600/400 spectrometer with tetramethylsilane (TMS) as an internal standard. MS spectra were obtained using a JEOL JMS-700/GI spectrometer and the waters UPLCMS system (Aquity UPLC XevoQTof). Column chromatography (CC) was performed on a neutral silica gel (Silica Gel 60 N, spherical, neutral, 40-50 μm) (KANTO Chemical Co., Inc.).

3.2.2. Extraction and isolation of chemical constituents from Stenochlaena palustris stems

The dried stems of *Stenoclaena palustris* (3.6 kg) were extracted at room temperature with methanol (MeOH). Removal of the solvent under reduced pressure afforded the extract (163.1 g). This extract was suspended in water and partitioned successively with *n*-hexane (*n*-Hex) and ethyl acetate (EtOAc) in this order, to give each fraction (*n*-Hex; 64.1 g and EtOAc; 30.3 g).

The EtOAc fraction (25.0 g) was divided by silica gel column chromatography (CC) eluting chloroform ($CHCl_3$) – MeOH step gradient polarity to yield 8 fractions (E1 to E8). E5 (3.5 g) was further purified by silica gel CC using $CHCl_3$/MeOH (1/0 to 0/1), to give 7 fractions (E5.1 to E5.7). E5.7 (487.1 mg) was purified by silica gel CC eluting $CHCl_3$/MeOH (8.5: 1.5) to yield compound **3** (57.1 mg). E7 (8.3 g) was purified by silica gel CC using $CHCl_3$/MeOH (1/0 to 0/1), to give 6 fractions (E7.1 - E7.6). E7.4 (3.1 g) was further purified by silica gel CC using $CHCl_3$/MeOH (9/1, 8.5/1.5 and 8/2), to give 5 fractions (E7.4.1 – E7.4.5). From E7.4.2 (487 mg) obtained compound **2** (245.8 mg). E7.4.4 (2.0 g) was purified by silica gel CC eluting $CHCl_3$/MeOH (8.5: 1.5) to yield compound **1** (1.9 g) (**Scheme 3**).



Scheme 3. Isolation scheme of *Stenochlaena palustris* stems extract.

3.2.3. Spectral data of isolated compounds

*20-Hydroxyecdysone (**1**)*

Yellow powder, HRESITOFMS *m/z* 503.2999 $[M + Na]^+$ (calcd. for $C_{27}H_{44}O_7Na$, 503.2985). ^1H NMR (400 MHz, CD_3OD): δ 5.81 (1H, d, *J* = 2.3 Hz, H-7), 4.88 (1H, s, H-3), 3.94 (1H, m, H-2), 3.84 (1H, dd, *J* = 11.7 and 1.8 Hz, H-22), 3.60 (1H, m, H-9), 3.33 (1H, dd, *J* = 14.2 and 1.4 Hz, H-5), 3.15 (1H, t, *J* = 8.3 Hz, H-17), 2.38 (1H, dd, *J* = 5.04 and 2.8 Hz, H-12), 2.15 - 1.26 (21H, m), 1.21 (3H, s, H-18), 1.20 (3H, s, H-21), 1.18 (3H, s, H-26), 0.96 (3H, s, H-27), 0.89 (3H, s, H-19); ^{13}C NMR (100 MHz, CD_3OD): δ 205.1, 166.7, 120.8, 83.9, 77.1, 76.6, 70.0, 67.4, 67.2, 50.4, 49.2, 48.5, 41.1, 38.0, 36.0, 33.8, 31.5, 31.2, 30.5, 28.4, 27.6, 26.0, 23.1, 20.2, 19.7, 16.7.

*Ajugasterone C (**2**)*

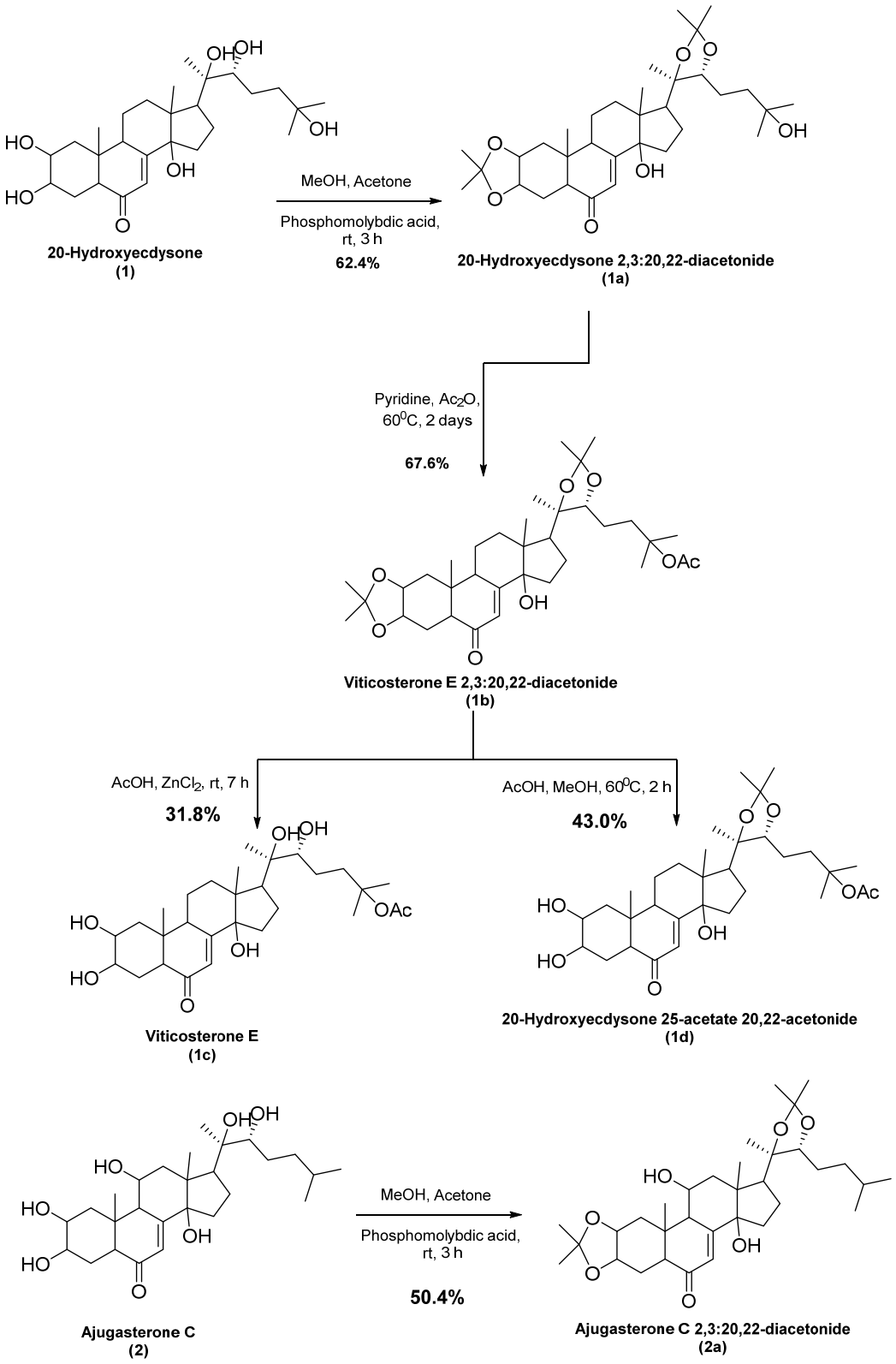
Yellow powder, HRESITOFMS *m/z* 503.2982 $[M + Na]^+$ (calcd. for $C_{27}H_{44}O_7Na$, 503.2985).^{1}H-NMR (400 MHz, CD_3OD): δ 5.80 (1H, d, *J* = 2.3 Hz, H-7), 4.88 (1H, s, H-3), 3.94 (1H, d, *J* = 3.2 Hz, H-2), 3.84 (1H, dd, *J* = 11.7 and 1.9 Hz, H-22), 3.60 (1H, m, H-9), 3.30 (1H, dd, *J* = 14.2 and 1.4 Hz, H-5), 3.14 (1H, t, *J* = 8.3 Hz, H-17), 2.38 (1H, m, H-12), 2.15 – 1.28 (21H, m), 1.2 (3H, s, H-18), 0.96 (3H, s, H-21), 0.95 (3H, s, H-26), 0.94 (3H, s, H-27), 0.88 (3H, s, H-19); ^{13}C NMR (100 MHz, CD_3OD): δ 205.1, 166.6, 120.8, 83.9, 76.4, 76.20, 76.1, 67.4, 67.2, 50.4, 49.1, 48.3, 47.1, 37.9, 36.0, 34.4, 33.8, 32.8, 31.5, 31.1, 30.43, 23.1, 20.2, 19.6, 18.0, 16.7, 15.6.

*Stenopalustroside A (**3**)*

Yellow powder, HRESITOFMS *m/z* 741.1803 $[M + H]^+$ (calcd. for $C_{39}H_{33}O_{15}$, 741.1819). ^1H NMR (400 MHz, CD_3OD): δ 7.99 (1H, d, *J* = 9.2 Hz, H-2'), 7.99 (1H, d, *J* = 9.1 Hz, H-6'), 7.70 (1H, d, *J* = 8.2 Hz, H-2'''), 7.70 (1H, d, *J* = 8.2, H-6'''), 7.47 (1H, d, *J* = 8.7 Hz, H-2''''), 7.47 (1H, d, *J* = 8.7 Hz, H-6''''), 6.89 (1H, d, *J* = 12.8, H-γ'''), 6.85 (1H, d, *J* = 9.6 Hz, H-3'), 6.85 (1H, d, *J* = 9.6, H-5'), 6.79 (1H, d, *J* = 7.8 Hz, H-3'''), 6.79 (1H, d, *J* = 7.8 Hz, H-5'''), 6.76 (1H, d, *J* = 12.8 Hz, H-γ''''), 6.75 (1H, d, *J* = 6.4 Hz, H-3''''), 6.75 (1H, d, *J* = 6.4 Hz, H-5''''), 6.26 (1H, d, *J* = 1.8 Hz, H-8), 6.12 (1H, d, *J* = 2.3 Hz, H-6), 5.88 (1H, d, *J* = 12.8 Hz, H-β'''), 5.54 (1H, d, *J* = 12.8 Hz, H-β''''), 5.38 (1H, d, *J* = 8.2 Hz, H-1''), 5.14 (1H, d, *J* = 9.6 Hz, H-3''), 4.23 (2H, m, H-6''), 3.67 (1H, *J* = 9.6 Hz and 7.8 Hz, H-2''), 3.58 (1H, m, H-5''), 3.34 (1H, d, *J* = 9.3 Hz, H-4''); ^{13}C NMR (100 MHz, CD_3OD): δ 177.9, 167.7, 167.5, 164.5, 161.5, 160.2, 159.9, 159.8, 157.8, 156.9, 145.5, 145.3, 133.9, 132.5, 130.9, 129.9, 126.0, 125.7, 121.3, 115.5, 115.4, 114.8, 114.5, 104.2, 102.6, 98.7, 93.6, 77.4, 74.4, 72.8, 68.8, 62.9.

3.2.4. Synthesis of 20-hydroxyecdysone derivatives

The synthesis of the 20-hydroxyecdysone derivatives followed the previous method with minor modifications (Suksamrarn and Pattanaprateep, 1995; Ves'kina and Odinokov, 2012). The scheme of synthesis of the 20-hydroxyecdysone derivatives was given in **Scheme 4**.



*Ves'kina and Odinokov, 2012. *Russian J. Org. Chem. 48*:1137-1161.
* Suksamrarn and Pattanaprateep, 1995. *Tetrahedron*. *51*:10633-10650.

Scheme 4. The synthesis scheme of the 20-hydroxyecdysone derivatives.

*20-Hydroxyecdysone 2,3:20,22-diacetonide (**1a**)*

Compound **1** (80 mg, 0.167 mmol) was dissolved in MeOH (1 ml) and dry acetone (15 ml, excess) added. Phosphomolybdic acid (8 mg *ca* 0.004 mmol) was added and the reaction mixture was stirred for 3 h. The mixture was neutralized with $NaHCO_3$ and the product extracted with $CHCl_3$. The organic phase washed with H_2O, dried over anhydrous Na_2SO_4, and purified by silica gel column chromatography using $CHCl_3$: MeOH (20:1) to give **1a** (58.2 mg, 62.4%).

Yellow powder, HRESITOFMS: *m/z* 561.3804 $[M + H]^+$ (calcd. for $C_{33}H_{53}O_7$, 561.3791). ^1H NMR (400 MHz, $CDCl_3$): δ 5.82 (1H, d, *J* = 2.28 Hz, H-7), 4.26 (1H, br s, H-3), 4.23 (1H, m, H-2), 3.65 (1H, d, *J* = 8.72 Hz, H-22), 2.81 (1H, m, H-9), 2.34 (1H, dd, *J* = 4.6 and 12.5 Hz, H-5), 2.22 (1H, t, *J* = 8.9 Hz, H-17), 2.07 – 1.53 (19H, m), 1.49, 1.41, 1.33 and 1.32 (12H, s, 2,3:20,22 diacetonide), 1.25 (3H, s, H-27 Me), 1.24 (3H, s, H-26 Me), 1.16 (3H, s, H-21 Me), 0.98 (3H, s, H-27 Me), 0.79 (3H, s, H-18 Me); ^{13}C NMR (100 MHz, $CDCl_3$): δ 203.1, 163.4, 121.4, 108.4, 107.1, 85.1, 84.5, 82.1, 76.8, 72.2, 71.7, 70.5, 50.9, 49.1, 47.5, 41.5, 37.9, 37.7, 34.6, 31.7, 31.0, 29.7, 29.3, 29.0, 28.6, 26.9, 26.7, 26.5, 23.7, 22.1, 21.3, 20.6, 17.1.

*Viticosterone E 2,3:20,22-diacetonide (**1b**)*

A mixture of **1a** (172.5 mg, 0.308 mmol), pyridine (1.44 ml) and Ac_2O (1.15 ml) was stirred for 2 days, 60ºC. The mixture was extracted with EtOAc and diluted with HCl 10%. The EtOAc phase was neutralized with $NaHCO_3$, dried over anhydrous Na_2SO_4 and purified by silica gel column chromatography using $CHCl_3$: MeOH (9:1) to give **1b** (127.2 mg, 67.6%).

Yellow powder, HRESITOFMS *m/z* 603.3873 $[M + H]^+$ (calcd. for $C_{35}H_{55}O_8$, 603.3897).). ^1H NMR (400 MHz, $CDCl_3$): δ 5.82 (1H, d, *J* = 2.32 Hz, H-7), 4.26 (1H, br s, H-3), 4.23 (1H, m, H-2), 3.60 (1H, dd, *J* = 8.04 and 3.2 Hz, H-22), 2.82 (1H, m, H-9), 2.34 (1H, dd, *J* = 12.8 and 5.04 Hz, H-5), 2.23 (1H, m, H-17), 2.10 – 1.51 (18H, m), 1.98 (3H, s, OAc), 1.49, 141, 133 and 132 (12H, s, 2,3;20,22-diacetonide), 147 (3H, s, H-27 Me), 144 (3H, s, H-26 Me), 1.15 (3H, s, H-21 Me), 0.98 (3H, s, H-19 Me), 0.79 (3H, s, H-18 Me); ^{13}C NMR (100 MHz, $CDCl_3$): δ 203.0, 170.7, 163.4, 121.4, 108.4, 107.1, 85.1, 84.2, 82.2, 81.5, 72.2, 71.7, 50.9, 49.2, 47.6, 38.5, 37.8, 37.7, 34.6, 31.7, 31.1, 29.1, 28.6, 27.1, 26.7, 26.5, 26.3, 25.8, 23.7, 23.4, 22.6, 22.0, 21.3, 20.6, 17.1.

*Viticosterone E (**1c**)*

1b (62.3 mg, 0.1034 mmol) in 70% AcOH (1.5 ml) was stirred for 1.5 h. $ZnCl_2$ (22.5 mg) was then added and the mixture was stirred for 7 h. The mixture was extracted with EtOAc and neutralized with $NaHCO_3$, dried over anhydrous Na_2SO_4, and purified by silica gel column chromatography using $CHCl_3$: MeOH (8:2) to give **1c** (17.2 mg, 31.8%).

Yellow powder, HRESITOFMS *m/z* 545.3088 $[M + Na]^+$ (calcd. for $C_{29}H_{46}O_8Na$, 545.3090). ^1H NMR (400 MHz, CD_3OD): δ 6.24 (1H, d, *J* = 2.2 Hz, H-7), 4.23 (1H, br s, H-3), 4.16 (1H, br d, *J* = 10.5 Hz, H-2), 3.83 (1H, br d, *J* = 10 Hz, H-22), 3.58 (1H, m, H-9), 3.02 (1H, dd, *J* = 13.2 and 3.5 Hz, H-5), 2.98 (1H, t, *J* = 9 Hz, H-17), 2.38 (1H, dd, *J* = 5.04 and 5.04 Hz, H-12), 1.96 (3H, s, OAc), 1.93 – 1.52 (23H, m), 1.46 (3H, s, H-21 Me), 1.43 (3H, s, H-27 Me), 1.39 (3H, s, H-26 Me), 1.21 (3H, s, H-18 Me), 1.05 (3H, H-19 Me) ; ^{13}C NMR (100 MHz, CD_3OD): δ

205.1, 166.7, 120.8, 83.9, 77.1, 76.6, 70.1, 67.4, 67.2, 50.4, 49.2, 48.5, 41.1, 37.9, 36.0, 33.8, 31.5, 31.2, 30.5, 28.4, 27.6, 26.1, 23.1, 20.2, 19.7, 16.7.

*2-Hydroxyecdysone 25-acetate 20,22-acetonide (**1d**)*

Acetic acid 70% (1 ml) was added to a solution of **1b** (69.5 mg, 0.114 mmol) in MeOH (0.25 ml) and then the mixture was stirred for 2 h at 60ºC. The mixture was extracted with $CHCl_3$, neutralized $NaHCO_3$, dried over anhydrous Na_2SO_4 and purified by silica gel column chromatography using $CHCl_3$: MeOH (9:2) to give **1d** (27.7 mg, 43.0%).

Yellow powder, HRESITOFMS *m/z* 585.3394 $[M + Na]^+$ (calcd. for $C_{32}H_{50}O_8Na$, 585.3403). ^1H NMR (400 MHz, $CDCl_3$): δ 5.86 (1H, d, *J* = 2.28 Hz, H-7), 4.05 (1H, br s, H-3), 3.7 (1H, m, H-2), 3.62 (1H, dd, *J* = 12.82 and 3.2 Hz, H-22), 3.49 (1H, m, H-9), 3.19 (1H, dd, *J* = 12.9 and 3.2 Hz, H-5), 2.5 (1H, t, *J* = 8.72 Hz, H-17), 1.98 (3H, s, OAc), 1.91 – 1.51 (13H, m), 1.47 (3H, s, H-21 Me), 1.44 (3H, s, H-26 Me), 1.42 (3H, s, H-27 Me), 1.41 and 1.30 (12H, s, 2,3;20,22-diacetonide), 1.15 (3H, s, H-19 Me), 0.98 (3H, s, H-18 Me); ^{13}C NMR (100 MHz, $CDCl_3$): δ 204.1, 170.7, 164.7, 121.6, 106.9, 84.9, 84.2, 82.2, 81.5, 76.7, 67.7, 67.4, 50.0, 49.2, 38.8, 38.4, 38.2, 36.8, 31.7, 31.1, 29.0, 28.9, 26.9, 26.2, 25.8, 23.9, 23.3, 22.5, 22.1, 21.2, 17.1.

*Ajugasterone C 2,3:20,22-diacetonide (**2a**)*

Compound **2** (160 mg, 0.333 mmol) was dissolved in MeOH (2 ml) and dry acetone (30 ml, excess). Phosphomolybdic acid (16 mg *ca* 0.008 mmol) was added and the reaction mixture was stirred for 3 h. The mixture was neutralized

with $NaHCO_3$ and the product was extracted with $CHCl_3$. The organic phase was washed with H_2O, dried over anhydrous Na_2SO_4, and purified by silica gel column chromatography using $CHCl_3$: MeOH (20:1) to give **2a** (94.1 mg, 50.4%).

Yellow powder, HRESITOFMS *m/z* 583.3622 $[M + Na]^+$ (calcd. for $C_{33}H_{52}O_7Na$, 583.3611). ^1H NMR (400 MHz, $CDCl_3$): δ 5.82 (1H, d, *J* = 2.28 Hz, H-7), 4.27 (1H, br s, H-3), 4.23 (1H, m, H-2), 3.82 (1H, dd, *J* = 10.1 and 3.24 Hz, H-22), 3.55 (1H, m, H-17), 2.81 (1H, m, H-9), 2.63 (1H, s, H-5), 2.34 (1H, dd, *J* = 12.8 and 5.04 Hz, H-12), 2.15 – 1.28 (18H, m), 1.49, 1.41, 1.34, and 1.33 (12H, s, 2,3;20,22-diacetonide), 1.25 (3H, s, H-27 Me), 1.16 (3H, s, H-26 Me), 0.98 (3H, s, H-21 Me), 0.89 (3H, s, H-19 Me), 0.79 (3H, s, H-18 Me); ^{13}C NMR (100 MHz, $CDCl_3$): δ 202.9, 163.2, 121.5, 108.4, 108.1, 85.1, 84.8, 81.8, 76.6, 72.2, 71.7, 69.6, 53.9, 50.9, 49.1, 47.5, 37.9, 37.6, 34.6, 33.8, 32.4, 31.8, 31.7, 31.0, 29.3, 29.1, 28.6, 27.1, 26.7, 26.5, 23.7, 22.2, 21.3, 20.6, 18.7, 17.6, 17.1.

3.2.5. UPLC-ESITOFMS procedures

The samples were dissolved in DMSO/H_2O (1/1) at 20 mg/mL and filtered through 0.45 μm membrane filter (ADVANTEC®, Japan) and an aliquot (5 μL) of the sample was injected in the UPLC. Analysis was carried out by the Waters UPLC system (Aquity UPLC XevoQTof), using a UPLC BEH C_{18} analytical column (1.7 μm, 2.1 × 100 mm). The mobile phase contained solvent A (1% v/v AcOH in distilled water) and solvent B (acetonitrile). The liner gradient system employed was: 0 - 30 min 90% solvent A to 70% solvent A and 10% solvent B to 30% solvent B; kept for 5 min; 35 - 45 min 70% solvent A to 50% solvent A and

30% solvent B to 50% solvent B. The column eluate was monitored at 260 nm UV absorbance. Positive mode was employed in ESITOFMS.

3.2.6. A no-choice test for antitermite activity

Termites

Workers and soldiers of *Coptotermes curvignathus* were captured around Bengkulu University, Bengkulu city, Indonesia, and kept in the dark room, which has been maintained at 28ºC ± 2ºC and 80% ± 5 of relative humidity (RH).

A no-choice test

A no-choice test was employed for evaluating antitermite activity according to the previous method with modifications (Chang and Cheng, 2002). The doses of MeOH extract, *n*-Hex and EtOAc fractions were prepared to 2%, and isolated compounds and 20-hydroxyecdysone derivatives were prepared to 1% (sample weight/filter paper weight × 100%). Samples were dissolved in 500 µL MeOH and were applied onto filter papers Whatman No. 1 (Ø 85 mm for MeOH extract, *n*-Hex and EtOAc fractions and Whatman No. 1, 21 x 21 mm for isolated compounds and 20-hydroxyecdysone derivatives). Filter paper without samples was used as the control and the filter papers were dried overnight in a vacuum desiccator. The treated filter paper was placed onto petri dishes (Ø 90 mm × 20 mm height) and 30 workers and 3 soldiers of *Coptotermes curvignathus* were added to each petri dish. The test dishes with covers were then placed on a wet cloths in plastic trays and kept in a dark room at room temperature (28ºC ± 2) and 80% ± 5 RH for two weeks. A few drops of water were periodically added to the

bottom edge of each petri dish. The number of dead termites was counted daily, and mass loss of filter paper disc was calculated at the end of the test. The antitermite activity was evaluated from the termite mortality (%) and mass loss of filter paper disc (%). Three replications were performed for each sample (Adfa et al., 2017).

3.3. Results and Discussion

3.3.1. Isolation of chemical constituents from Stenochlaena palustris stems

The crude methanol extract (163.1 g) of *S. palustris* stems was partitioned successively with *n*-hexane and then EtOAc, to yield *n*-Hex (64.1 g) and EtOAc (30.3 g) fractions. At a dose of 2% samples for 7 days, antitermite effects of the MeOH extract, *n*-Hex and EtOAc fractions were assessed with the no-choice test against *C. curvignathus* (30 workers and 3 soldiers). Paper disc consumption by the termites after exposure to samples was compared to that of the control, and the average termite mortality and disc mass losses are shown in **Figures 32 and 33**. Termites were all dead from treatment of the MeOH extract and EtOAc fraction within 6 days (**Figure 32**). In contrast, the *n*-Hex fraction showed relatively low termicidal activity. In mass losses of paper discs, the MeOH extract and EtOAc fraction also exerted powerful influence on feeding behavior relative to *n*-Hex fraction. We therefore attempted to isolate chemical constituents responsible for antitermite properties from the EtOAc fraction.

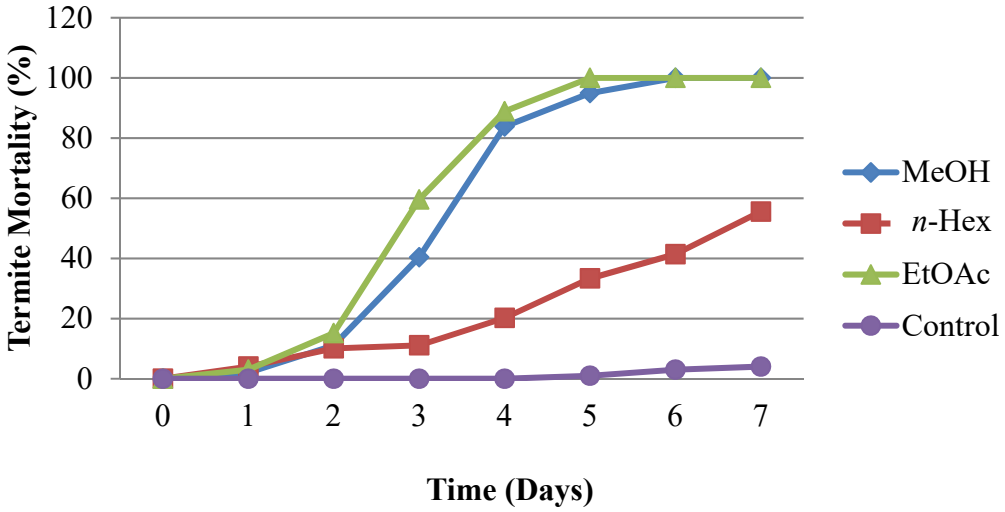


Figure 32. Antitermite activity of fractions from *Stenochlaena palustris* stems against *Coptotermes curvignathus,* dose 2%, (Means, *n* = 3).

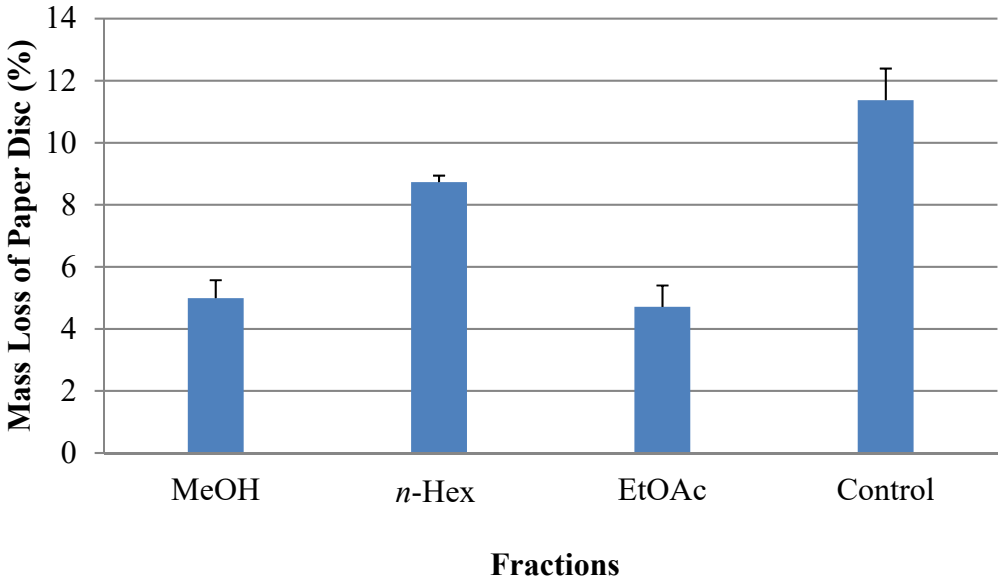


Figure 33. Paper disc consumption by *Coptotermes curvignathus* after 7 days exposure to *Stenochlaena palustris* stems fractions, dose 2%, (Means ± SEMs, *n* = 3).

The EtOAc fraction were separated and purified by CC on silica gel (SiO2) to yield three compounds. The chemical structures of the isolated compounds

were elucidated by their ^{1}H, ^{13}C and 2D NMR spectra of each compound and literature data. The isolated compounds from *S. palustris* stems are summarized in **Figure 34**.

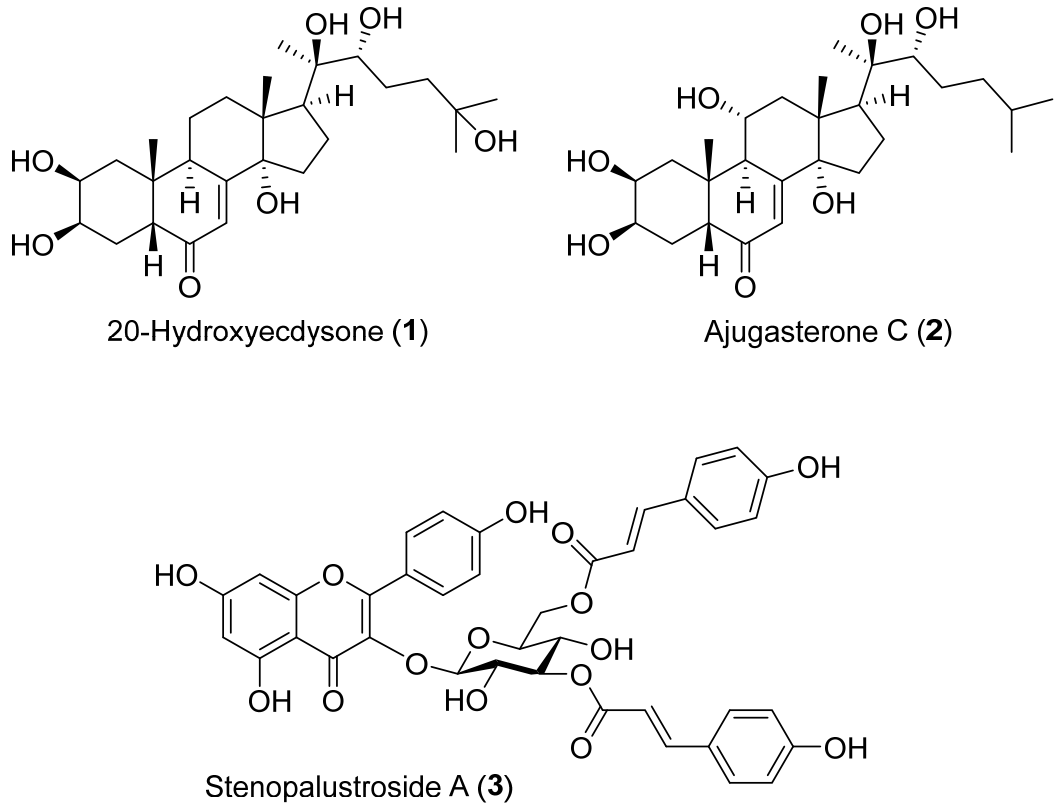


Figure 34. The chemical structures of isolated compounds from *Stenochlaena palustris* stems.

The HRESITOFMS peak at *m/z* 503.2999 $[M + Na]^{+}$ (calcd. for $C_{27}H_{44}O_7Na$, 503.2985) suggested $C_{27}H_{44}O_7$ as the molecular formula of compound **1**. Characteristically, its ^{1}H NMR displayed an olefinic proton at δ_H 5.81 (1H, d, J = 2.3 Hz, H-7), five methyl signals at δ_H 1.21 (3H, s, H-18), 1.20 (3H, s, H-21), 1.18 (3H, s, H-26), 0.96 (3H, s, H-27), and 0.89 (3H, s, H-19), and three oxymethine

proton signals at δ_H 4.88 (1H, s, H-3), 3.94 (1H, m, H-2), and 3.84 (1H, dd, *J* = 11.7 and 1.8 Hz, H-22). In ^{13}C NMR, a carbonyl carbon at δ_C 205.1, olefinic carbons at δ_C 166.7 and 120.8, and six oxygenated carbons at δ_C 83.9, 77.1, 76.6, 70.0, 67.4, and 67.2, and another 18 carbons were observed. Detailed 1D and 2D NMR analysis determined the chemical structure of **1** as 20-hydroxyecdysone. The key ^1H - ^1H COSY and HMBC correlation of compound **1** is shown in **Figure 35** and the NMR spectra data of compound **1** comparing with literature data was given in **Table 11**.

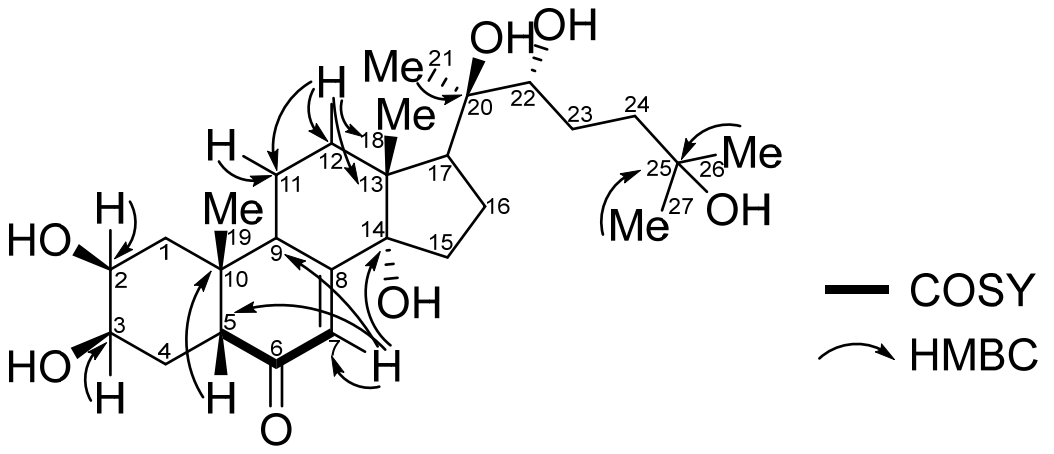


Figure 35. Key ^1H - ^1H COSY and HMBC correlation of compound **1**.

Table 11. ^{1}H and ^{13}C NMR data of compound **1**.

No	Compound **1** (CD_3OD)		20-hydroxyecdysone (C_5D_5N)*	
	δ_C	δ_H	δ_C	δ_H
1	36.0		38.0	
2	67.4	3.94, m	68.3	4.15, m
3	67.2	4.88, brs	68.2	4.20, brs
4	31.5		32.5	
5	50.4	3.33, m	51.4	2.97, m
6	205.1		203.5	
7	120.8	5.81, d, J = 2.3 Hz	121.7	6.22, d, J = 2.1 Hz
8	166.7		166.1	
9	33.8	3.60, m	34.6	3.55, m
10	38.0		38.8	
11	19.7		21.2	
12	31.2		32.1	
13	48.5		48.2	
14	83.9		84.4	
15	31.2		31.8	
16	20.2		21.6	
17	49.2	3.15, m	50.2	2.96, m
18	16.7	1.21, s	17.9	1.18, s
19	26.0	0.89, s	24.5	1.02, s
20	76.6		77.0	
21	23.1	1.20, s	21.7	1.56, s
22	77.1	3.84, d, J = 8.2 Hz	77.7	3.84, d, J = 8.9 Hz
23	27.6		27.5	
24	41.1		42.6	
25	70.0		69.8	
26	28.4	1.18, s	30.1	1.34, s
27	30.5	0.96, s	30.2	1.34, s

*Suksamrarn and Pattanaprateep, 1995. *Tetrahedron 51*:10633-10650.

The ^{1}H NMR spectrum of compound **2** had a remarkable similarity to that of compound **1** in the signals arising from the protons on the A, B, and D rings. The striking differences, compared to **1**, were the absence of an oxygenated quaternary signal in the side-chain and appearance of an oxymethine signal on the C ring. The chemical structure of **2** was determined to be ajugasterone C (Suksamrarn and Pattanaprateep, 1995) (**Figure 36** and **Table 12**).

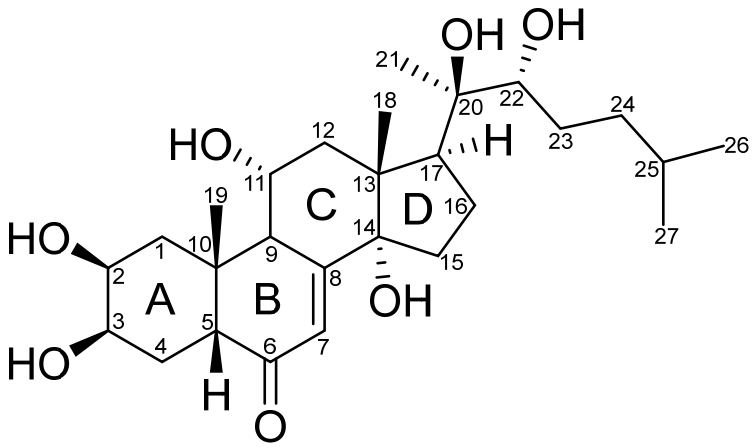


Figure 36. The chemical structure of ajugasterone C (**2**).

Table 12. ^{1}H and ^{13}C NMR data of ajugasterone C (**2**).

No	Ajugasterone C (**2**) (CD_3OD)	
	δ_C	δ_H
1	36.0	
2	67.4	3.94, d, *J* = 3.2 Hz
3	67.2	4.88, s
4	33.8	
5	50.4	3.30, dd, *J* = 14.2, 1.4 Hz
6	205.1	
7	120.8	5.80, d, *J* = 2.3 Hz
8	166.6	
9	34.4	3.60, s
10	37.9	
11	76.1	
12	32.8	2.38, m
13	48.3	
14	83.9	
15	32.8	
16	18.7	
17	49.1	3.14, t, *J* = 8.3 Hz
18	16.7	1.20, s
19	20.2	0.88, s
20	76.2	
21	19.6	0.96, s
22	76.4	3.84, dd, *J* = 11.7, 1.9 Hz
23	23.1	
24	47.1	
25	16.7	
26	30.43	0.95, s
27	30.5	0.94, s

The ^{1}H and ^{13}C NMR of compound **3** possessed the characteristics of kaempferol glucoside and two *p*-coumaroyl moieties. The HMBC analysis indicated that one *p*-coumaroyl unit located at the C-3" position and another *p*-coumaroyl unit attached at the C-6" position on the glucoside, respectively. Detailed spectroscopic analyses and comparison with data reported previously (Liu et al., 1999), **3** was confirmed as stenopalustroside A (**Figure 37** and **Table 13**).

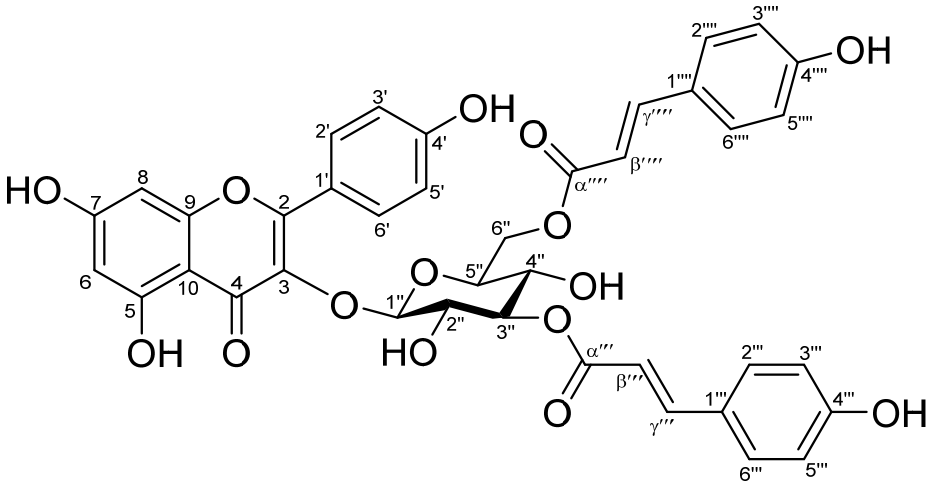


Figure 37. The chemical structure of stenopalustroside A (**3**).

Table 13. ^{1}H and ^{13}C NMR data of compound **3**.

No	Compound **3** (CD_3OD)		Stenopalustroside A (CD_3OD)*	
	δ_C	δ_H	δ_C	δ_H
2	157.8		159.4	
3	133.9		135.1	
4	177.9		179.3	
5	161.5		163.1	
6	98.70	6.12, d, *J* = 2.3 Hz	100.1	6.20, d, *J* = 2.1 Hz
7	164.5		166.4	
8	93.60	6.26, d, *J* = 1.8 Hz	94.9	6.32, d, *J* = 2.1 Hz
9	156.9		158.5	
10	104.2		105.6	
1'	121.3		122.7	
2'	132.5	7.99, d, *J* = 9.2 Hz	132.2	7.96, d, *J* = 8.9 Hz
3'	115.4	6.85, d, *J* = 9.6 Hz	116.1	6.84, d, *J* = 8.9 Hz
4'	160.2		161.5	
5'	115.4	6.85, d, *J* = 9.6 Hz	116.1	6.84, d, *J* = 8.9 Hz
6'	132.5	7.99, d, *J* = 9.2 Hz	132.2	7.96, d, *J* = 8.9 Hz
1''	102.6	5.38, d, *J* = 8.2 Hz	103.8	5.32, d, *J* = 7.9 Hz
2''	72.8	3.67, dd, *J* = 9.6, 7.8 Hz	74.0	3.61, dd, *J* = 9.3, 7.9 Hz
3''	77.4	5.14, d, *J* = 9.6 Hz	78.3	5.09, t, *J* = 9.3 Hz
4''	68.8	3.34, d, *J* = 9.3 Hz	70.0	3.47, t, *J* = 9.3 Hz
5''	74.4	3.58, m	75.5	3.52, m
6''	62.9	4.23, m	63.8	4.22, m
α'''	167.7		167.9	
β'''	115.5	5.88, d, *J* = 12.8 Hz	116.8	5.88, d, *J* = 12.8 Hz
γ'''	145.3	6.89, d, *J* = 12.8 Hz	144.9	6.89, d, *J* = 12.8 Hz
1'''	126.0		127.6	
2'''	132.5	7.70, d, *J* = 8.2 Hz	133.7	7.68, d, *J* = 8.6 Hz
3'''	114.8	6.79, d, *J* = 7.8 Hz	115.8	6.75, d, *J* = 8.6 Hz
4'''	159.9		160.0	
5'''	114.8	6.79, d, *J* = 7.8 Hz	115.8	6.75, d, *J* = 8.6 Hz
6'''	132.5	7.70, d, *J* = 8.2 Hz	133.7	7.68, d, *J* = 8.6 Hz
α''''	167.5		167.7	
β''''	115.4	5.54, d, *J* = 12.8 Hz	116.1	5.53, d, *J* = 12.8 Hz
γ''''	145.5	6.76, d, *J* = 12.8 Hz	145.5	6.72, d, *J* = 12.8 Hz
1''''	125.7		127.6	
2''''	132.5	7.47, d, *J* = 8.7 Hz	133.7	7.50, d, *J* = 8.6 Hz
3''''	114.5	6.75, d, *J* = 6.4 Hz	115.8	6.69, d, *J* = 8.6 Hz
4''''	159.8		160.0	
5''''	114.5	6.75, d, *J* = 6.4 Hz	115.8	6.69, d, *J* = 8.6 Hz
6''''	132.5	7.47, d, *J* = 8.7 Hz	133.7	7.50, d, *J* = 8.6 Hz

*Liu et al., 1999. *J. Nat. Prod. 62*:70-75.

3.3.2. Antitermite activity of isolated compounds from Stenochlaena palustris stems

The antitermite effects against *Coptotermes curvignathus* were tested with paper discs impregnated with each isolated compounds at a dose of 1% for 7 days. Results of antitermite responses are summarized in **Figures 38 and 39**. The three isolates greatly affected mortality and feeding of 33 termites. All compounds killed off the termites within 6 days. Mass losses of paper discs were 6.29% for **1**, 9.23% for **2**, and 7.16% for **3**. Notably, treatment of 20-hydroxyecdysone (**1**) had a highly significant effect on termite mortality and mass loss of paper disc. These data clearly identified that the isolated three compounds **1**-**3** were causative agents with respect to the antitermite properties of *S. palustris* stems.

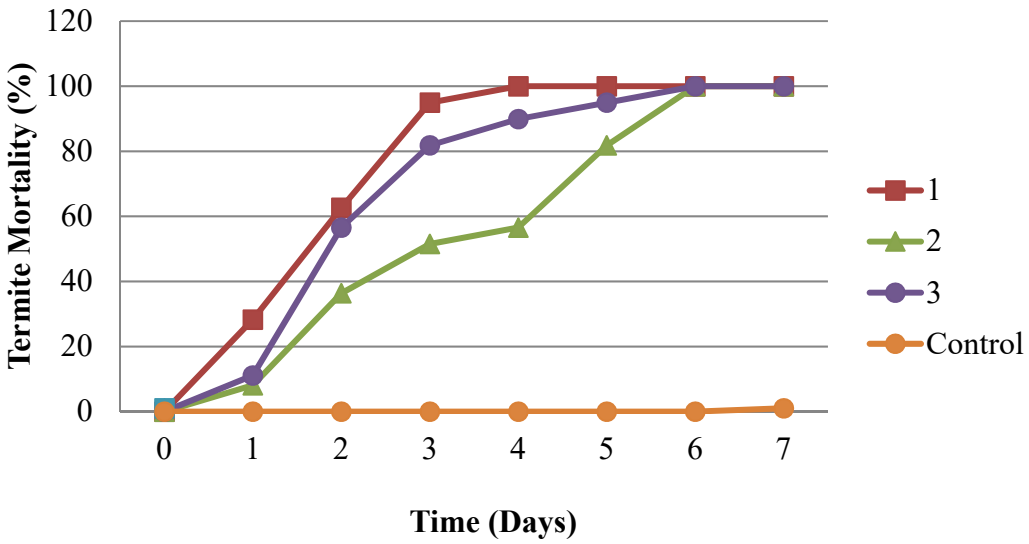


Figure 38. Termite mortality by treatment of isolated compounds from *Stenochlaena palustris* stems against *Coptotermes curvignathus,* dose 1%, (Means, *n* = 3). 20-Hydroxyecdysone (**1**), ajugasterone C (**2**) and stenopalustroside A (**3**).

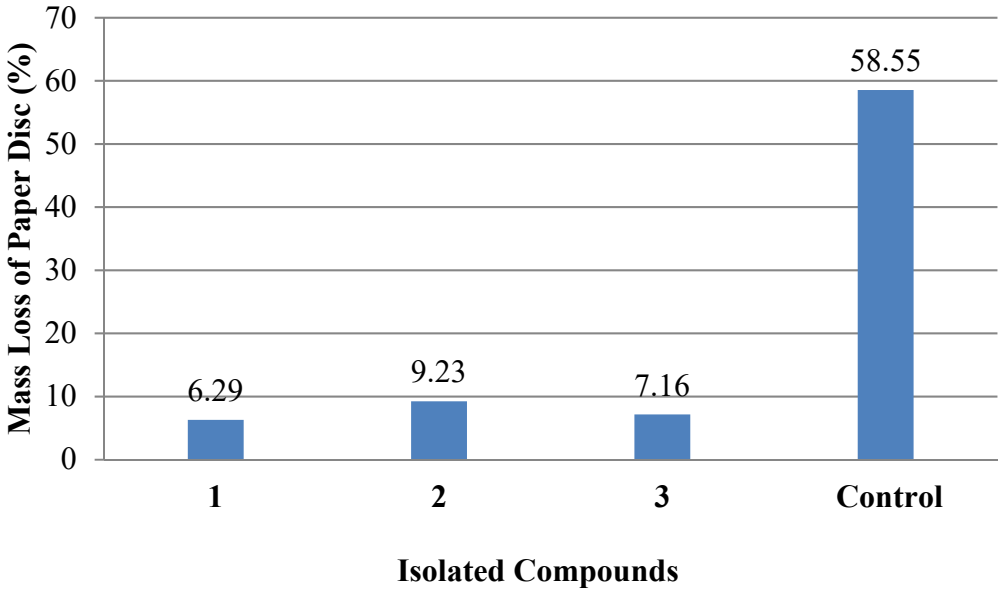


Figure 39. Paper disc consumption by *Coptotermes curvignathus* after 7 days exposure to isolated compounds from *Stenochlaena palustris* stems. 20-Hydroxyecdysone (**1**), ajugasterone C (**2**) and stenopalustroside A (**3**). Dose 1%, (Means ± SEMs, $n = 3$).

3.3.3. UPLC-ESITOFMS analysis

UPLC-ESITOFMS analysis of chemical constituents in EtOAc fraction of *S. palustris* stems was carried out. A UPLC BEH C_{18} RP column, gradient elution (1% AcOH (aq)/acetonitrile), and 260 nm detection were selected (Pardede et al., 2017). The UPLC-ESITOFMS analytical chromatogram is shown in **Figure 40**. The retention time of each compound in the EtOAc fraction was assigned 15.72, 21.88 and 42.13 min for 20-hydroxyecdysone (**1**), ajugasterone C (**2**), and stenopalustroside A (**3**), respectively. This protocol revealed that the isolates are major constituents in the EtOAc fraction of *S. palustris* stems. The overwhelming evidence here also supported compounds **1** - **3** as causes for antitermite activity of

S. palustris stems. The HRESITOFMS spectrum of each retention time of compounds isolated from *Stenochlaena palustris* stems were given in **Figure 41**.

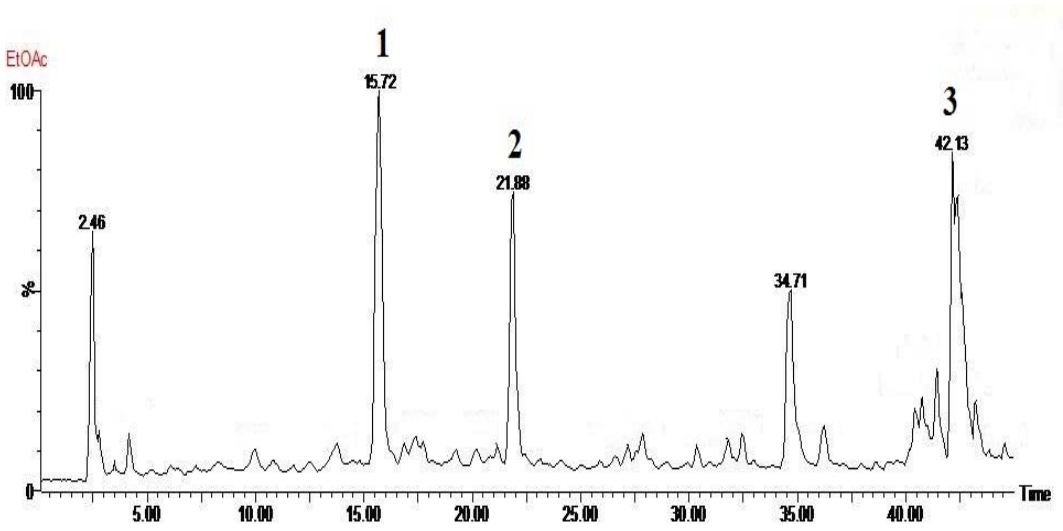


Figure 40. UPLC-ESITOFMS chromatogram of the EtOAc fraction of *Stenochlaena palustris* stems. 20-Hydroxyecdysone (**1**), ajugasterone C (**2**) and stenopalustroside A (**3**).

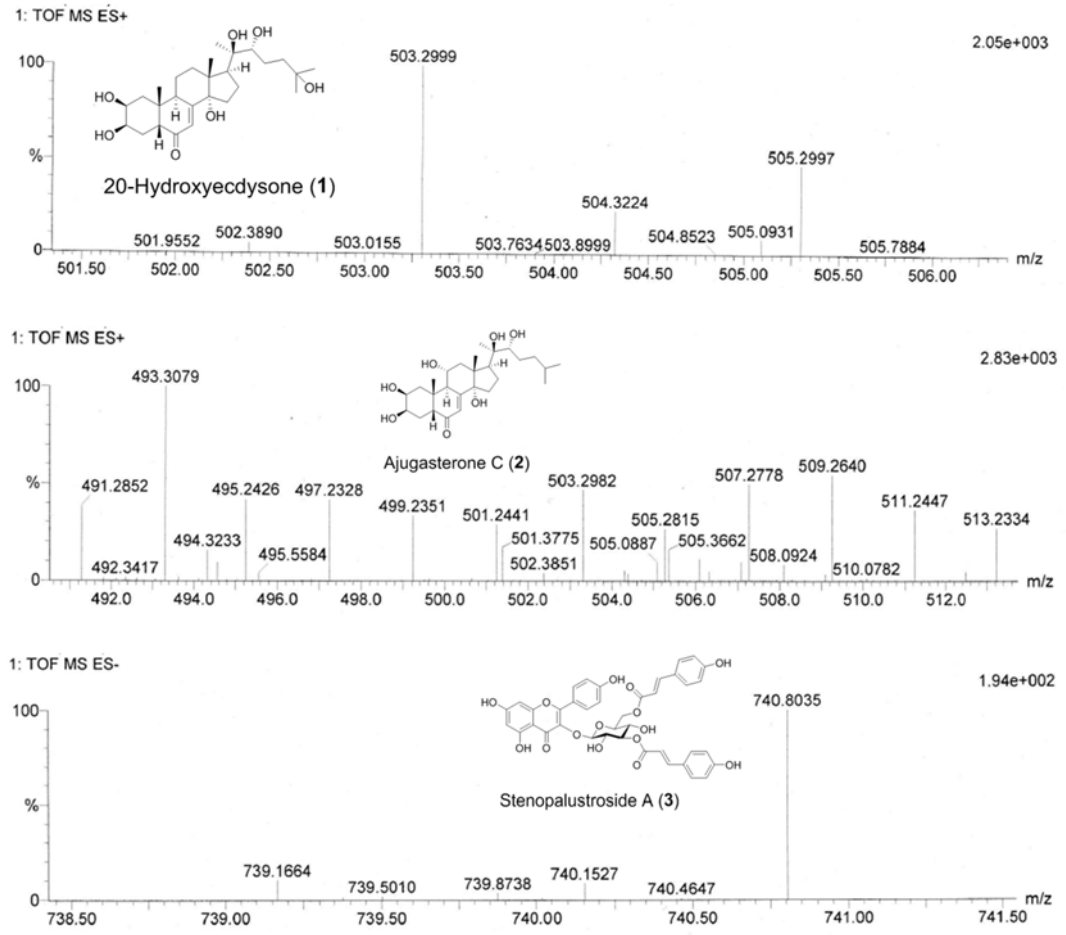


Figure 41. The HRESITOFMS spectrum of each retention time of compounds isolated from *Stenochlaena palustris* stems.

3.3.4. Structure activity relationships (SAR) of 20-hydroxyecdysone derivatives on antitermite activity

In insects, ecdysteroids are the major steroid hormones involved in regulating growth and development. Especially, 20-hydroxyecdysone (**1**) is known as a molting hormone. Su and Monteagudo reported that 20-hydroxyecdysone (**1**) showed high mortality against both *Coptotermes formosanus* Shiraki (Formosan subterranean termite) and *Reticulitermes flavipes* (Kollar) (Eastern subterranean

termite) in the 12-day no-choice test (Su and Monteagudo, 2017). As described above, 20-hydroxyecdysone (**1**) exerted a drastic effect on termites. This ability of 20-hydroxyecdysone (**1)** is attributable to the presence of hydroxy groups. Next step focuses on verifying the importance of the degree of hydroxylation of 20-hydroxyecdysone responsible for the antitermite properties. In order to establish which hydroxyl groups in the 20-hydroxyecdysone and the derivatives are the most beneficial in promoting antitermite effects, hydroxy groups were sequentially protected. By reference to previous reports (Suksamrarn and Pattanaprateep, 1995; Suksamrarn and Yingyongnarongkul, 1996; Ves'kina and Odinokov, 2012), 20-hydroxyecdysone2,3:20,22-diacetonide (**1a**), viticosterone E 2,3:20,22-diacetonide (**1b**), viticosterone E (**1c**), and 20-hydroxyecdysone25-acetate 20,22-acetonide (**1d**) were synthesized from **1** *via* acetonidation, *O*-acetylation, and selective deprotection. In addition, ajugasterone C 2,3:20,22-diacetonide (**2a**) was prepared from **2**. Their chemical structures are illustrated in **Figure 42**. Detailed synthetic procedures and physical data of the derivatives are given in Materials and Methods section.

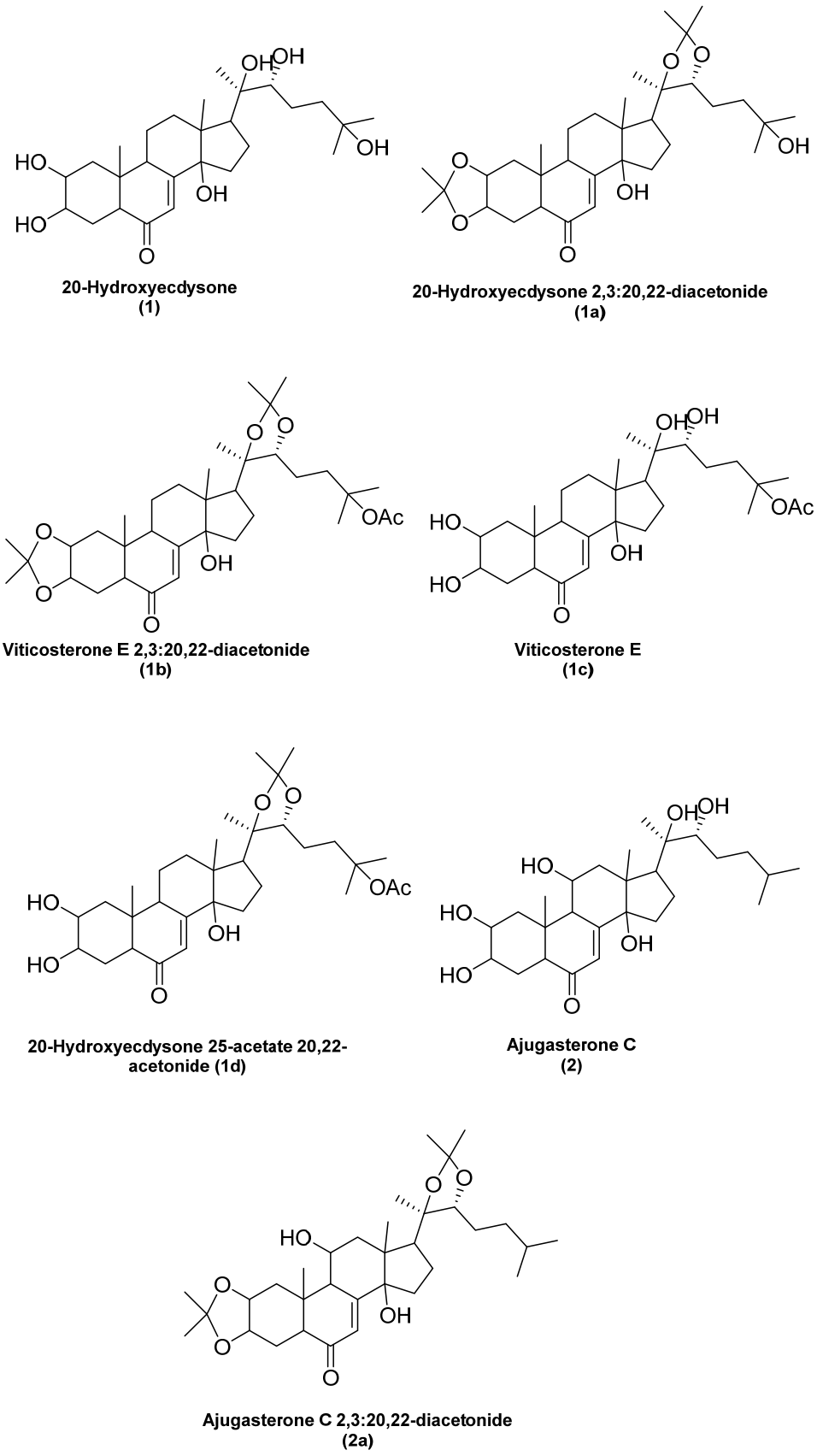


Figure 42. Chemical structures of 20-hydroxyecdysone derivatives.

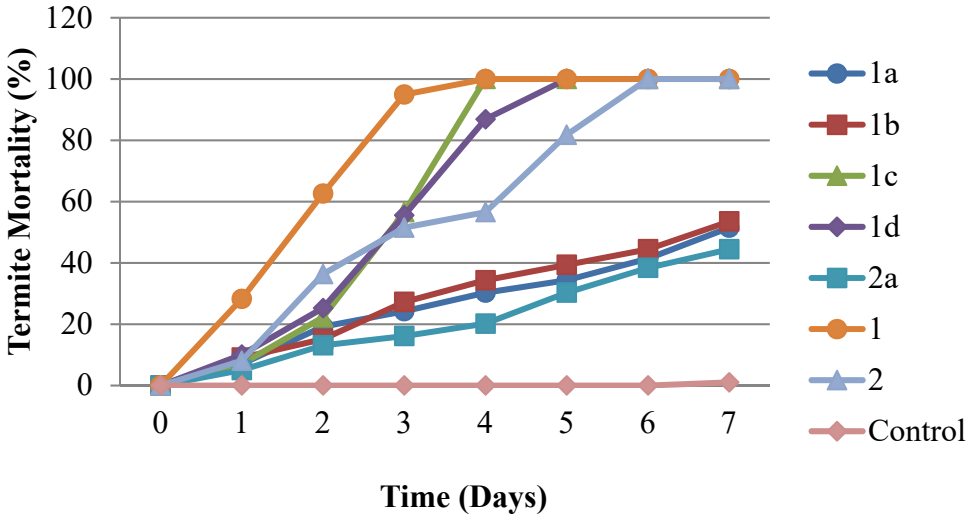


Figure 43. Termite mortality by treatment of 20-hydroxyecdysone derivatives against *Coptotermes curvignathus,* dose 1%, (Means, *n* = 3). 20-Hydroxyecdysone 2,3:20,22-diacetonide (**1a**), viticosterone E 2,3:20,22-diacetonide (**1b**), viticosterone E (**1c**), 20-hydroxyecdysone 25-acetate 20,22-acetonide (**1d**), ajugasterone C 2,3:20,22-diacetonide (**2a**), 20-hydroxyecdysone (**1**) and ajugasterone C (**2**).

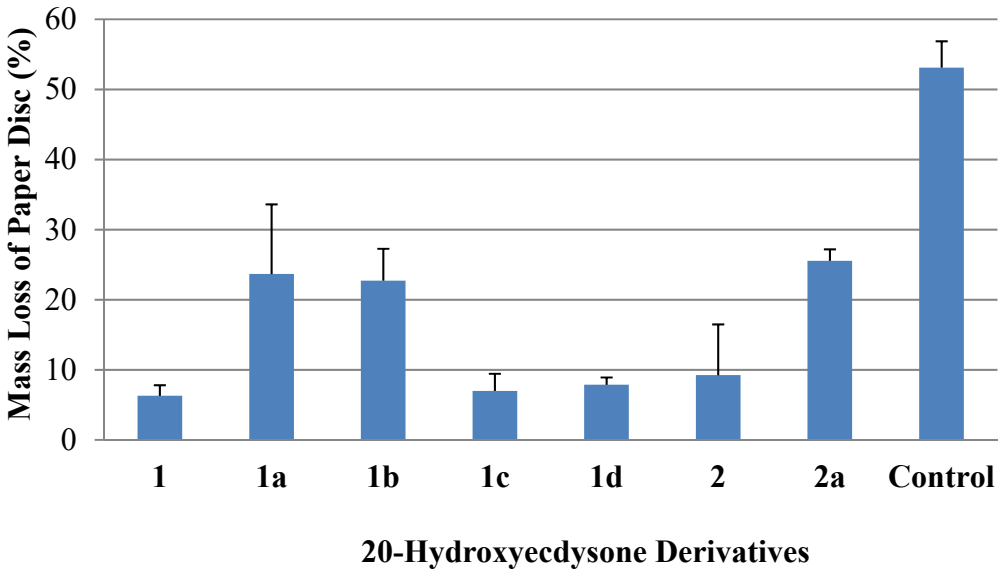


Figure 44. Paper disc consumption by *Coptotermes curvignathus* after 7 days exposure to 20-hydroxyecdysone derivatives. 20-Hydroxyecdysone 2,3:20,22-doacetonide (**1a**), viticosterone E 2,3:20,22-diacetonide (**1b**), viticosterone E (**1c**), 20-hydroxyecdysone 25-acetate 20,22-acetonide (**1d**), ajugasterone C 2,3:20,22-diacetonide (**2a**), 20-hydroxyecdysone (**1**) and ajugasterone C (**2**), dose 1%, (Means ± SEMs, *n* = 3).

As indicated in **Figures 43 and 44**, and all derivatives showed antitermite properties (termite mortality and mass losses of paper discs) against *C. curvignathus* at a dose of 1%. Among the derivatives tested, **1c** and **1d** were active comparable to intact **1**. Compared to them, the derivatives **1a** and **1b** showed relatively weak activity. Comparing with **2a** to **2**, a similar tendency was denoted. These findings exemplified that a 2,3-diol is the most influential, and a 20,22-diol and a 25-OH group carry very little weight on antitermite activity of 20-hydroxyecdysone (**1**).

3.4. Conclusions

In our endeavor to explore the antitermite constituents from *S. palustris* stems, we concluded that 20-hydroxyecdysone (**1**), ajugasterone C (**2**) and stenopalustroside A (**3**) are mainly responsible for the efficacy. More attention must be given because *S. palustris* stems possessed excellent potential as a natural resource of ecdysteroids. On the basis of scientific evidence in this study, our results will broaden the field of the application of *S. palustris* stems as effectives for controlling termite population.

References

Adfa, M., Kusnanda, A. J., Livandri, F., Rahmad, R., Darwis, W., Efdi, M., Ninomiya, M., Koketsu, M. 2017. Insecticidal activity of *Toona sinensis* against *Coptotermes curvignathus* Holmgren. *Rasayan J. Chem. 10*:153-159.

Adfa, M., Livandri, F., Meita, N. V., Manaf, S., Ninomiya, M., Gustian, I., Putranto, A. M. H., Supriati, R., Koketsu, M. 2015. Termitical activity of *Acorus calamus* Linn, rhizomes and its main constituents against *Coptotermes curvignathus* Holmgren. *J. Asia Pac. Entomol. 18*:47-50.

Chai, T. T., Kwek, M. T., Ong, H. C., Wong, F. C. 2015. Water fraction of edible medicinal fern *Stenochlaena palustris* is a potent α-glucosidase inhibitor with concurrent antioxidant activity. *Food Chem. 186*:26-31.

Chang, S. T., Cheng, S. S. 2002. Antitermite activity of leaf essential oil and compounds from *Cinnammum asmophleum*. *J. Agric. Food 50*:1389-1392.

Chear, N. J. Y., Khaw, K. Y., Murugaiya,h, V., Lai, C. S. 2016. Cholinesterase inhibitory activity and chemical constituents of *Stenochlaena palustris* fronds at two different stages of maturity. *J. Food Drug. Anal. 24*:358-366.

Giron, M. Y, San Pablo, M. R. 2004. Resistance of some forest vines to wood-destroying fungi. *FPRDI Journal, 26*:58-67.

Liu, H., Orjala, J., Rali, T., Sticher, O. 1998. Glycosides from *Stenochlaena palustris*. *Phytochemistry 49*:2403-2408.

Liu, H., Orjala, J., Sticher, O., Rali, T. 1999. Acylated flavonol glycosides from leaves of *Stenochlaena palustris. J. Nat. Prod. 62*:70-75.

Mamadalieva, N. Z., El-Readi, M. Z., Janibekov, A. A., Tahrani, A., Wink, M. 2011. Phytoecdysteroids of *Silene guntensis* and their in vitro cytotoxic and antioxidant activity. *Z. Naturforsch 66c*:215-224.

Oramahi, H. S., Yoshimura, T. 2013. Antifungal and antitermite activities of wood vinegar from *Vitex pubesscens* Vahl. *J. Wood Sci. 59*:344-350.

Pardede, A., Adfa, M., Kusnanda, A. J., Ninomiya, M., Koketsu, M. 2017. Flavonoid rutinosides from *Cinnamomum parhenoxylon* leaves ant their hepatoprotective and antioxidant activity. *Med. Chem. Res. 26*:2074-2079.

Roszaini, K., Nor Azah, M. A., Mailina, J., Zaini, S., Faridz, Z. M. 2013. Toxicity and antitermite activity of the essential oils from *Cinnamomum camphora, Cymbopogon nardus, Melaleuca cajuputi and Dipterocarpus sp.* against *Coptotermes curvignathus. Wood Sci. Technol. 47*:1273-1284.

Suksamrarn, A., Pattanaprateep, P. 1995. Selective acetylation of 20-hydroxyecdysone partial synthesis of some minor ecdysteroids and analogues. *Tetrahedron 51*:10633-10650.

Suksamrarn, A., Yingyongnarongkul, B. 1996. Synthesis and biological activity of 2-deoxy-20-hydroxyecdysone and derivatives. *Tetrahedron 52*:12623-12630.

Su, NY, Monteagudo, EJ., 2017. Hyperecdysonism in the Formosan subterranean termite and Eastern subterranean termite (Isoptera: Rhinotermitidae). *J. Economic. Entomol. 110*:1736-1739.

Swmya, SN., Calistus, J. A. L., Deepa, M. 2016. antitermite properties of four selected species of Zingiberaceae extracts. *J. Zool. St. 3*:24-29.

Ves'kina, N. A., Odinokov, V. N. 2012. Transformations of ecdysteroids in the synthesis of their structural analogs and minor phytoecdysteroids. *Russian J. Org. Chem. 48*:1137-1161.

Zuraini, Z., Sasidharan, S., Kaur, S. R., Nithiyayini, M. 2010. Antimicrobial and antifungal activities of local edible fern *Stenochlaena palustris* (Burm. F) Bedd. *Pharmacologyonline 1*:233-237.

Chapter 4

Chemical constituents of *Coreopsis lanceolata* stems and their antitermite activity against *Coptotermes curvignathus*

4.1. Introduction

Termites are one of the widely prevailing pests that inflict economic damage on agricultural crops. They are also known to cause tremendous loss to the finished and unfinished wooden structures in buildings (Sowmya et al., 2016; Mishra et al., 2017). Termites create colonies, consists of several types of termites such as eggs, nymphs, workers, soldiers, alates and reproductives (king and queen). The termite life cycle were given in **Figure 45**.

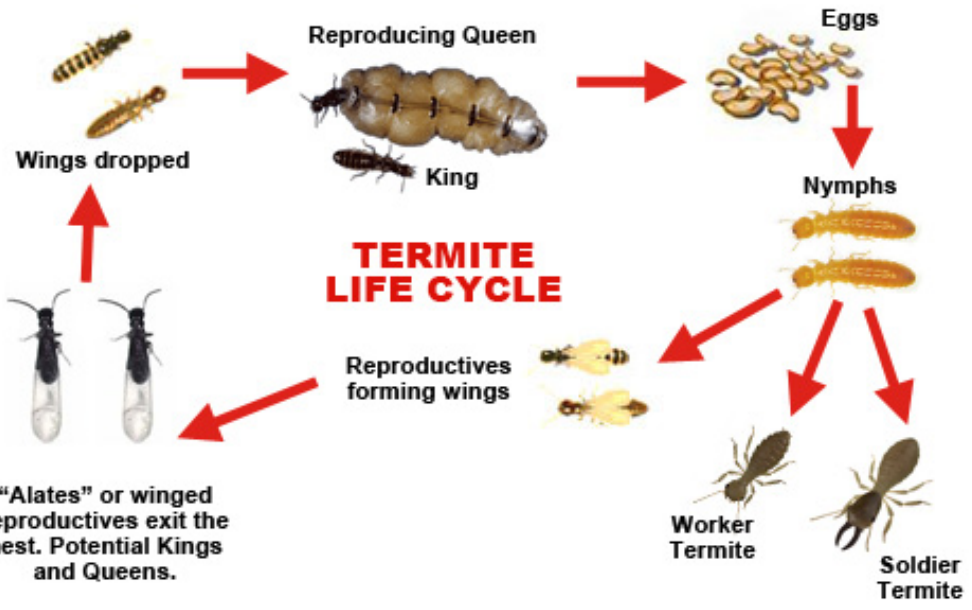
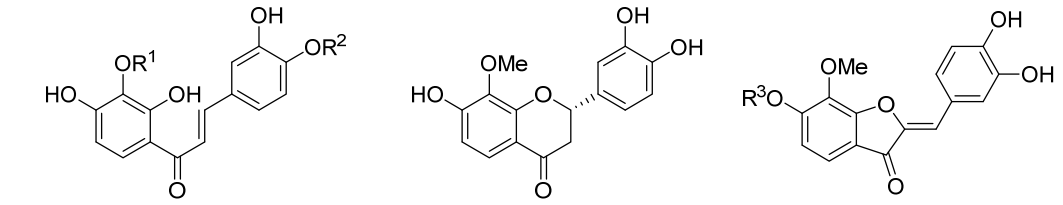


Figure 45. Termite life cycle (http://lifeafterbugs.com/termite-treatment/).

Coptotermes curvignathus belongs to the *Rhinotermitidae* family, this termite species being mainly responsible for wood destruction in countries such as Indonesia and Malaysia. With the aim of termite control, numerous synthetic termiticidal agents have been developed, however, they cause environmental pollution and residual problems. Much attention has been given recently to the discovery of the natural alternatives for termite control (Chan et al., 2011; Oramahi and Yoshimura 2014; Adfa et al., 2017).

Coreopsis lanceolata is a perennial plant that belongs to the *Asteraceae* family. Previous phytochemical studies reported that chemical constituents isolated from various parts of *C. lanceolata* exerted interesting biological activities. For instance, flavonoids from *C. lanceolata* flower petals showed useful antioxidant activity (Tanimoto et al., 2009; Shang et al., 2013; Okada et al., 2014). Effective nematicidal molecules isolated from the flowers and sepals have been reported against *Bursaphelenchus xylophilus, Pratylenchus penetrans*, and *Caenorhabditis elegans* (Kimura et al., 2008). Recently, we identified that rare flavonoids including a flavonone, chalcones, and aurones were major components of the flowers and they displayed potent antileukemic properties (**Figure 46**) (Pardede et al., 2016). The picture of *Coreopsis lanceolata* is show in **Figure 47**.



R^1 = Me, R^2 = H: **Lanceoletin** (**1**)
R^1 = R^2 = H: **Okanin** (**2**)
R^1 = Me, R^2 = Me: **4-Methoxylanceoletin** (**3**)

8-Methoxybutin (**4**)

R^3 = H: **Leptosidin** (**5**)
R^3 = Glc: **Leptosin** (**6**)

Figure 46. Chemical structures of flavonoids isolated from *Coreopsis lanceolata* flowers.

a b c d

Figure 47. The picture of *Coreopsis lanceolata*, flowers (a), leaves (b), stems (c), and dried stems has been chopped (d).

However, there has been no investigation of *C. lanceolata* stems on antitermite activity against *Coptotermes curvignathus.* Our efforts have been

directed to search for the possibilities of natural compounds as termite control agents (Hiramatsu et al., 2013; Ninomiya et al., 2014; Adfa et al., 2017). Within the scope of our ongoing program, we explored antitermite agents from *C. lanceolate* stems.

4.2. Materials and Methods

4.2.1. General experimental procedures

^{1}H and ^{13}C NMR spectra were recorded on a JEOL ECA 400 spectrometer (JEOL Ltd, Tokyo, Japan) with tetramethylsilane (TMS) as an internal standard. MS spectra were obtained using the Waters UPLC-MS system (Aquity UPLC XevoQTof) (Waters Corporation, Milford, USA). Column chromatography (CC) was performed on a neutral silica gel (Silica Gel 60 N, spherical, neutral, 40-50 μm) (KANTO Chemical Co., Inc, Tokyo, Japan).

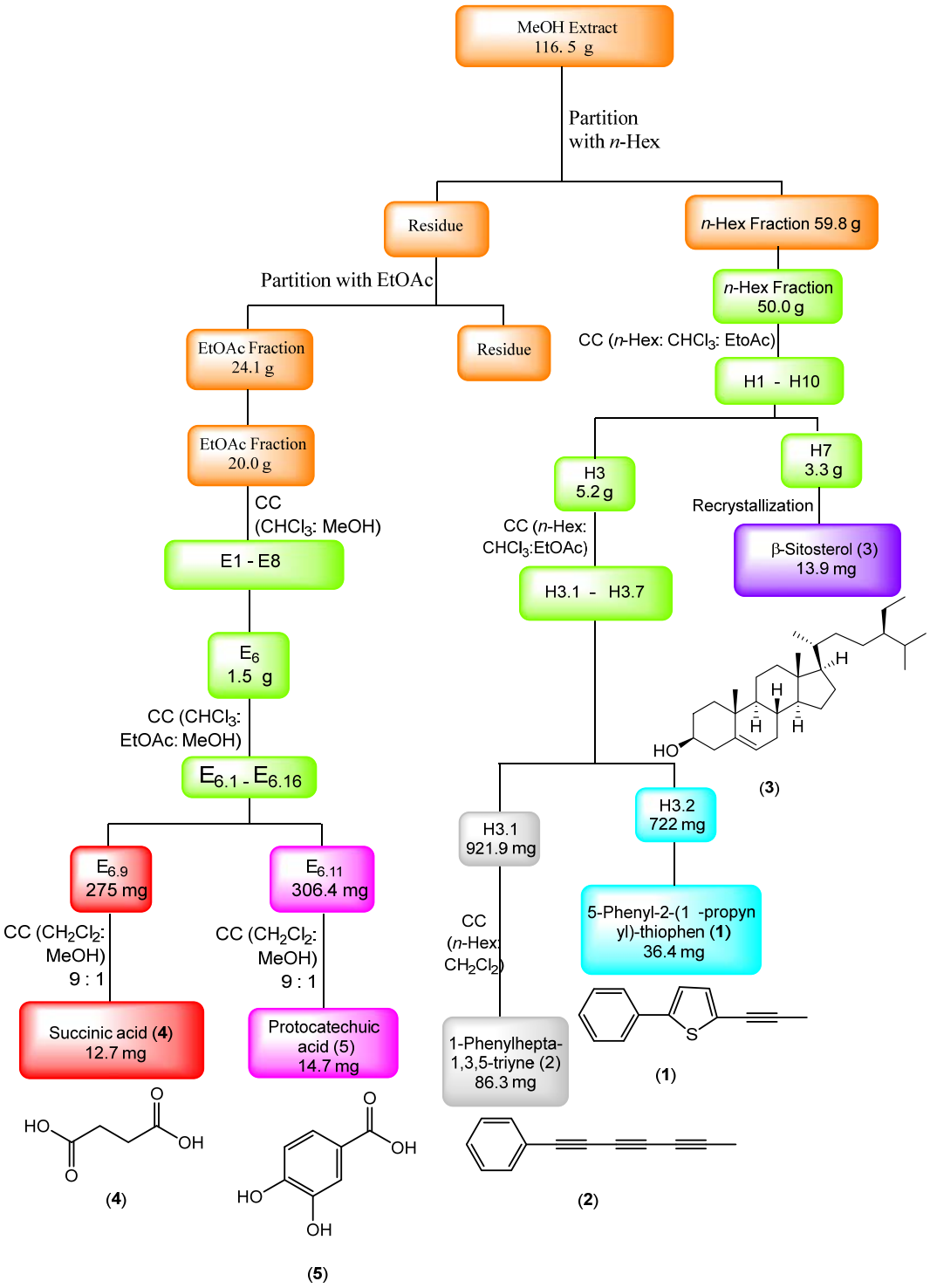
4.2.2. Extraction and isolation of chemical constituents from Coreopsis lanceolata stems

The dried stems of *C. lanceolata* (2.8 kg) were extracted at room temperature with methanol (MeOH). Removal of the solvent under reduced pressure afforded the extract (116.5 g). This extract was suspended in water and partitioned successively with *n*-hexane (*n*-Hex) and ethyl acetate (EtOAc) in this order, to give each fraction (*n*-Hex; 59.8 g and EtOAc; 24.1 g).

The *n*-Hex fraction (50.0 g) was divided by silica gel column chromatography (CC) eluting *n*-Hex - chloroform ($CHCl_3$) - EtOAc step gradient polarity to yield 10 fractions (H1 to H10). H3 (5.2 g) was further purified by silica

gel CC using *n*-Hex/$CHCl_3$ (1/0 to 0/1) and then $CHCl_3$/EtOAc (1/0 to 0/1), to give 7 fractions (H3.1 to H3.7). From H3.2 (722 mg) obtained compound **1** (36.4 mg). H3.1 (921.9 mg) was purified by silica gel CC eluting *n*-Hex/dichloromethane (CH_2Cl_2) to yield compound **2** (86.3 mg). Compound **3** (13.9 mg) obtained from H7 (3.3 g) by recrystallization.

The EtOAc fraction (20.0 g) was divided by silica gel CC eluting $CHCl_3$/MeOH (1/0 to 0/1) to yield 8 fractions (E1 to E8). E6 (1.5 g) was further purified by silica gel CC using $CHCl_3$/EtOAc (1/0 to 0/1) and then EtOAc/MeOH (1/0 to 0/1), to give 16 fractions (E6.1 to E6.16). E6.9 (275 mg) was purified by silica gel CC eluting (CH_2Cl_2: MeOH 9:1) to yield compound **4** (12.7 mg). E6.11 (306.4 mg) was purified by silica gel CC eluting (CH_2Cl_2: MeOH 9:1) to yield compound **5** (14.7 mg) (**Scheme 5**).



Scheme 5. Isolation scheme of *Coreopsis lanceolata* stems extract.

4.2.3. Spectral data of isolated compounds

*5-Phenyl-2-(1-propynyl)-thiophene (**1**)*

Yellow oil, HRESITOFMS *m/z* 199.0594 $[M+H]^+$ (calcd for $C_{13}H_{10}S$, 199.0581). ^1H NMR (400 MHz, $CDCl_3$): δ 7.55 (2H, d, *J* = 7.3 Hz, H-2" and H-6"), 7.36 (2H, dd, *J* = 7.3 Hz and 7.8 Hz, H-3" and H-5"), 7.27 (1H, d, *J* = 7.36 Hz and 9.6 Hz, H-4"), 7.13 (1H, d, *J* = 3.64 Hz, H-3), 7.07 (1H, d, *J* = 4.12 Hz, H-4), 2.09 (3H, s, H-3'); ^{13}C NMR (100 MHz, $CDCl_3$): δ 144.5, 133.9, 132.1, 129.0, 127.8, 125.9, 123.5, 122.8, 90.9, 73.2, 4.8.

*1-Phenylhepta-1,3,5-triyne (**2**)*

Yellow oil, ^1H NMR (400 MHz, $CDCl_3$): δ 7.50 (2H, dd, *J* = 6.9 Hz and 1.84 Hz, H-2' and H-6'), 7.35 (1H, dd, *J* = 7.32 Hz and 8.72 Hz, H-4'), 7.31 (2H, dd, *J* = 1.84 Hz and 6.88 Hz, H-3' and H-5'), 2.00 (3H, s, H-7); ^{13}C NMR (100 MHz, $CDCl_3$): δ 133.1, 129.6, 128.6, 121.2, 78.4, 75.3, 74.7, 67.5, 65.0, 59.1, 4.8.

*β-Sitosterol (**3**)*

Colorless needle crystals, HREIMS m/z 414.9071 $[M]^+$ (indicating a molecular formula $C_{29}H_{50}O$). ^1H NMR (400 MHz, $CDCl_3$): δ 5.35 (1H, t, *J* = 2.7 Hz, H-5), 5.12 - 4.99 (2H, m, H-22 and H-23), 3.59 - 3.48 (1H, m, H-3), 2.17 - 2.31 (1H, m, H-20), 2.06 - 1.81 (10H, m), 1.71 - 1.44 (9H, m), 1.43 - 1.35 (4H, m), 1.13 - 1.07 (3H, m), 1.06 - 0.91 (6H, m, H-19 and H-29), 0.86 - 0.77 (9H, m, H24, H-26, and H-27), 0.73- 0.67 (3H, m, H-28); ^{13}C NMR (100 MHz, $CDCl_3$): δ 140.8, 121.8, 71.8, 56.8, 56.1, 50.3, 50.2, 45.9, 42.4, 42.3, 39.8, 37.3, 36.5, 36.2, 34.0, 31.9, 31.7, 29.2, 28.3, 26.1, 24.3, 23.1, 21.1, 19.9, 19.4, 19.1, 18.8, 12.0, 11.9.

*Succinic acid (**4**)*

Colorless needle crystals, ^{1}H NMR (400 MHz, CD_3OD): δ 2.56 (4H, s, H-2 and H-3); ^{13}C NMR (100 MHz, CD_3OD): δ 174.86, 28.54.

*Protocatechuic acid (**5**)*

White needle crystals, HRESITOFMS *m/z* 153.0180 $[M-H]^-$ (calcd for $C_7H_6O_4$, 153.0188). ^{1}H NMR (400 MHz, CD_3OD): δ 7.43 (1H, d, *J* = 2 Hz, H-6), 7.41 (1H, d, *J* = 2.3 Hz, H-2), 6.79 (1H, d, *J* = 8.7 Hz, H-5); ^{13}C NMR (100 MHz, CD_3OD): δ 168.9, 150.2, 144.7, 122.3, 121.8, 116.4, 114.4.

4.2.4. A no-choice test for Antitermite Activity

Termites

Workers and soldiers of *Coptotermes curvignathus* were obtained around Bengkulu University, Bengkulu city, Indonesia and kept in the dark room, which has been maintained at 28ºC ± 2ºC and 80% ± 5 of relative humidity (RH).

A no-choice test

A no-choice test was employed for evaluating antitermite activity according to the previous method with modification (Chang and Cheng 2002). The dose of MeOH extract, *n*-Hex and EtOAc fractions were prepared to 1% and 2%, and isolated compounds were prepared to 1%, 0.5% and 0.25% (sample weight/filter paper weight × 100%). Samples were dissolved in 500 µL MeOH and were applied onto filter papers Whatman No. 1 (Ø 85 mm for MeOH extract, *n*-Hex and EtOAc fractions and Whatman No. 1, 21 x 21 mm for isolated compounds). Filter paper

without samples was used as the control and the filter papers were dried overnight in a vacuum desiccator. The treated filter paper was placed onto petri dishes (Ø 90 mm × 20 mm height) and 30 workers and 3 soldiers of *Coptotermes curvignathus* were added to each petri dish. The test dishes with covers were then placed on a wet cloths in plastic trays and kept in a dark room at room temperature (28ºC ± 2) and 80% ± 5 RH for two weeks. A few drops of water were periodically added to the bottom edge of each petri dish. The number of dead termites was counted daily, and mass loss of filter paper was calculated at the end of the test. The antitermite activity was evaluated from the termite mortality (%) and mass loss of filter paper disc (%). Three replications were performed for each sample (Adfa et al. 2017).

4.3. Results and Discussion

4.3.1. Isolation of chemical constituents from Coreopsis lanceolata stems

The antitermite activity of plant extracts has been reported to find the possibilities of natural compounds as termite control agents (Raje et al. 2015). Investigation for antitermite activity of *Coreopsis lanceolata* stems extract was conducted. The crude methanol (MeOH) extract (116.5 g) of *Coreopsis lanceolata* stems was partitioned successively with *n*-hexane (*n*-Hex) and ethyl acetate (EtOAc), to yield *n*-Hex fraction (59.8 g) and EtOAc fraction (24.1 g), respectively. At two doses of 1% and 2% samples, antitermite effects of the MeOH extract, *n*-Hex and EtOAc fractions were assessed with the no-choice test against *C. curvignathus* (30 workers and 3 soldiers) for 14 days. Paper disc consumption by the termites was compared to that of the control at the end of the test. The average termite mortality

and paper disc consumption are shown in **Figures 48** and **49**. The termite mortality of MeOH extract, *n*-Hex and EtOAc fractions at dose 1% were 98.1%, 100%, and 81.8%, respectively. Furthermore, the termite mortality of MeOH extract, *n*-Hex, and EtOAc fractions at a dose of 2% were 100%, 100% and 95.1%, respectively. In the mass losses of paper discs, the same tendency was denoted. According to the results of antitermite activity of MeOH extract, *n*-Hex and EtOAc fractions, we therefore attempted to isolate chemical constituents responsible for antitermite properties from the *n*-Hex and EtOAc fractions.

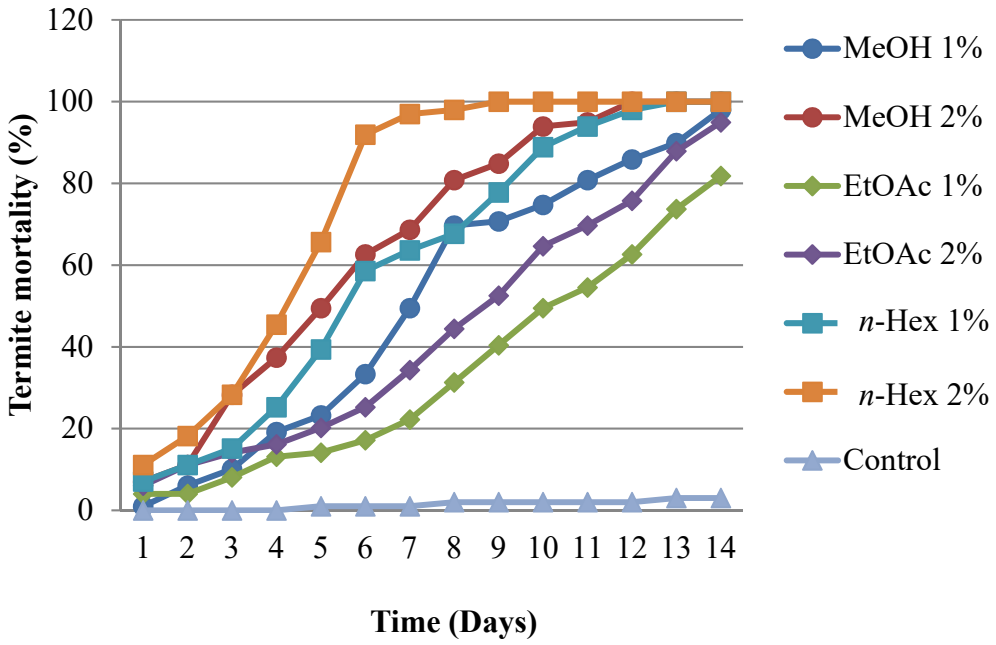


Figure 48. Termite mortality by treatment of fractions from *Coreopsis lanceolata* stems against *Coptotermes curvignathus,* doses 1% and 2%, (Means, *n* = 3).

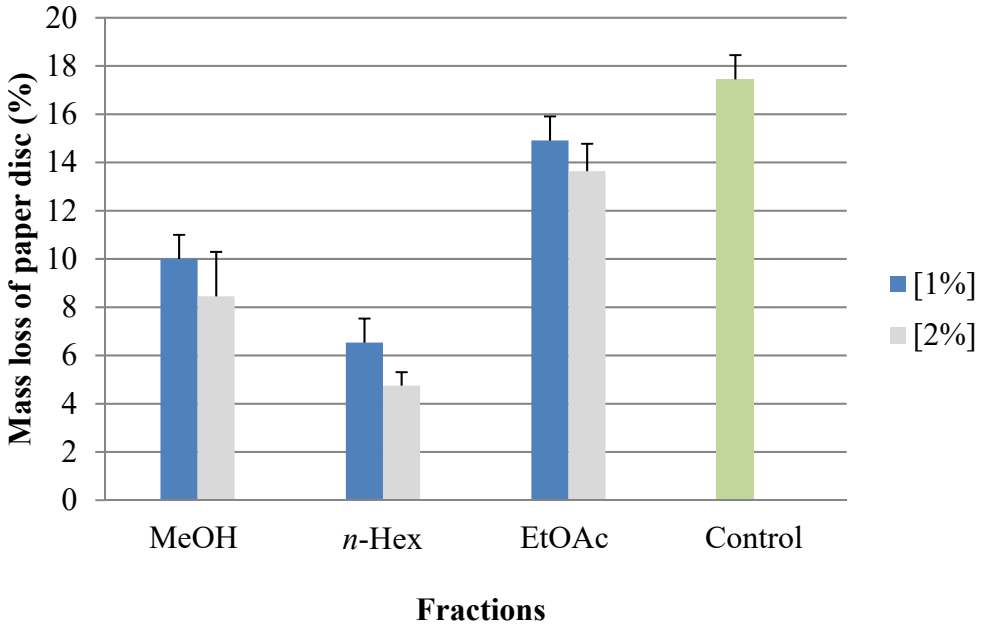


Figure 49. Paper disc consumption by *Coptotermes curvignathus* after 14 days exposure to fractions of *Coreopsis lanceolata* stems, doses 1% and 2%, (Means ± SEMs, $n = 3$).

Compound **1** was isolated as yellow oil, and its molecular formula was established as $C_{13}H_{10}S$ from HRESITOFMS *m/z* 199.0594 $[M+H]^+$(calculated for $C_{13}H_{11}S$, 199.0581). Its ^1H and ^{13}C NMR displayed the signals of an aromatic ring, a triple bond, and a methyl groups. Thiophene signals at δ_H 7.13 (1H, d, $J = 3.6$ Hz, H-3) and 7.07 (1H, d, $J = 4.1$ Hz, H-4) were observed. These data determined the chemical structure of **1** as 5-phenyl-2-(1-propynyl)-thiophene (**Figure 50** and **Table 14**).

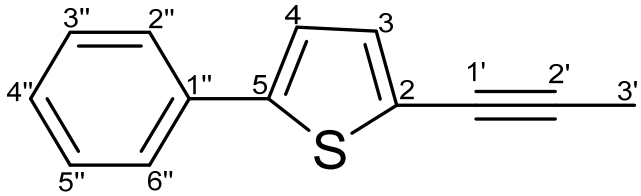


Figure 50. The chemical structure of compound **1**.

Table 14. ^{1}H and ^{13}C NMR data of compound **1**.

No	Compound **1** (**(**$CDCl_3$)		5-Phenyl-2-(1-propynyl)-thiophene ($CDCl_3$)*	
	δ_C	δ_H	δ_C	δ_H
2	144.5		144.4	
3	122.8	7.13, d, *J* = 3.6 Hz	122.7	7.13, d, *J* = 3.7 Hz
4	132.1	7.07, d, *J* = 4.1 Hz	132.0	7.07, d, *J* = 3.7 Hz
5	123.5		123.4	
1'	73.2		73.1	
2'	90.9		90.8	
3'	4.8	2.09, s	4.7	2.09, s
1"	133.9		133.9	
2"	125.9	7.55, d, *J* = 7.3 Hz	125.8	7.55, d, *J* = 7.3 Hz
3"	129.0	7.36, dd, *J* = 7.8, 7.3 Hz	128.9	7.36, dd, *J* = 7.8, 7.3 Hz
4"	127.8	7.27, d, *J* = 7.4 Hz	127.7	7.27, br d, *J* = 7.8 Hz
5"	129.0	7.36, dd, *J* = 7.8, 7.3 Hz	128.9	7.36, dd, *J* = 7.8, 7.3 Hz
6"	125.9	7.55, d, *J* = 7.3 Hz	125.8	7.55, d, *J* = 7.3 Hz

*Kimura et al., 2008. *Z Naturforsch 63c*:843-847.

Compound **2** is yellow oil. The ^{1}H NMR spectrum showed the presence of aromatic proton at δ_H 7.50 (2H, dd, *J* = 6.9 and 1.84 Hz), 7.35 (1H, dd, *J* = 7.32 and 8.72 Hz) and 7.31 (2H, dd, *J* = 1.84 and 6.88 Hz) were assigned to H-2', H-6', H-4', H-3' and H-5', respectively. Instead of thiophene signals, additional triple bond characteristics were appeared in ^{13}C NMR spectrum at δ_C 78.4, 75.3, 74.7,

67.5, 65.0, and 59.1 in compound **2** resulted in the structural elucidation as 1-Phenylhepta-1,3,5-triyne (**Figure 51** and **Table 15**).

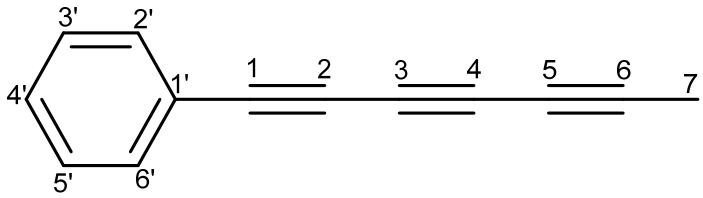


Figure 51. The chemical structure of compound **2**.

Table 15. ^{1}H and ^{13}C NMR data of compound **2**.

No	Compound **2** (CDCl$_3$)		1-Phenylhepta-1,3,5-triyne (CDCl$_3$)*	
	δ_C	δ_H	δ_C	δ_H
1	75.3		75.2	
2	74.7		74.2	
3	59.0		58.9	
4	67.5		67.4	
5	65.0		64.9	
6	78.4		78.3	
7	4.8	2.00, s	4.7	2.00, s
1'	121.2		121.1	
2'	133.1	7.50, dd, *J* = 6.9, 1.8 Hz	132.9	7.50, dd, *J* = 7.7, 1.2 Hz
3'	129.6	7.31, dd, *J* = 6.9, 1.8 Hz	129.5	7.31, dd, *J* = 7.7, 7.2 Hz
4'	128.6	7.35, dd, *J* = 7.3, 8.7 Hz	128.4	7.37, dd, *J* = 7.2, 1.2 Hz
5'	129.6	7.31, dd, *J* = 6.9, 1.8 Hz	129.5	7.31, dd, *J* = 7.7, 7.2 Hz
6'	133.1	7.50, dd, *J* = 6.9, 1.8 Hz	132.9	7.50, dd, *J* = 7.7, 1.2 Hz

*Kimura et al., 2008. *Z Naturforsch 63c*:843-847.

Compound **3** was isolated as colorless needle crystals. The molecular formula was established as $C_{29}H_{50}O$ from HREIMS *m/z* 414.9071 $[M]^+$. The spectral data of compound **3** were identical to those previously reported (Kamboj

and Saluja, 2011; Chaturvedula and Prakash, 2012; Pardede and Koketsu, 2017), **3** was confirm as β-sitosterol (**Figure 6** and **Table 3**).

Compound **4** was obtained as colorless needle crystals. The ^{13}C NMR spectrum showed the presence of 2 atom carbons. The ^{1}H and ^{13}C NMR spectra were in good agreement with the reported data (El-attar et al., 2015), compound **4** was confirmed to be succinic acid (**Figure 52 and Table 16**).

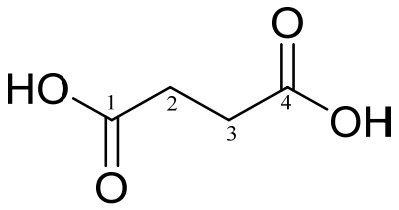


Figure 52. Chemical structure of succinic acid (**4**).

Table 16. ^{1}H and ^{13}C NMR data of compound **4**.

No	Compound **4** (CD_3OD)		Succinic acid (CD_3OD)*	
	δ_C	δ_H	δ_C	δ_H
1	174.9		173.6	
2	28.5	2.56, s	28.7	2.41, s
3	28.5	2.56, s	28.7	2.41, s
4	174.9		173.6	

*El-attar et al., 2015. *World J. Pharm. Sci. 3*: 689-695.

Compound **5** was isolated as white needle crystals and its molecular formula was established as $C_7H_6O_4$ from HRESITOFMS *m/z* 153.0180 $[M-H]^-$ (calcd for $C_7H_5O_4$, 153.0188). The ^{1}H NMR spectrum showed the aromatic proton signals at δ_H 7.43 (1H, d, *J* = 2 Hz), 7.41 (1H, d, *J* = 2.3 Hz) and 6.79 (1H, d, *J* = 8.7 Hz) were assigned to H-6, H-2 and H-5, respectively. The ^{13}C NMR spectrum displayed 7 carbon signals, 1 carbonyl carbon at δ_C 168.9. The HRESITOFMS, ^{1}H

and ^{13}C NMR data confirmed that **5** was protocatechuic acid (**Figure 53** and **Table 17**) (Azzizudin et al, 2010).

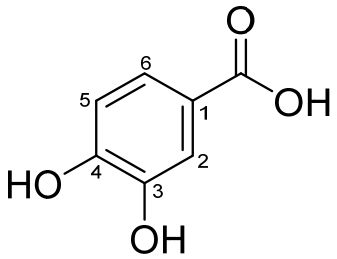


Figure 53. Chemical structure of protocatechuic acid (**5**).

Table 17. ^{1}H and ^{13}C NMR of protocatechuic acid (**5**).

No	Protocatechuic acid (**5**)	
	δ_C	δ_H
1	121.8	
2	116.4	7.41 d, J = 2.3 Hz
3	144.7	
4	150.2	
5	114.4	6.79 d, J = 8.7 Hz
6	122.3	7.43, d, J = 2 Hz
C=O	168.9	

Using chromatographic techniques, five constituents were isolated from *Coreopsis lanceolata* stems. Their chemical structures were confirmed by spectroscopic analysis. Their chemical structures are depicted in Figure **54.**

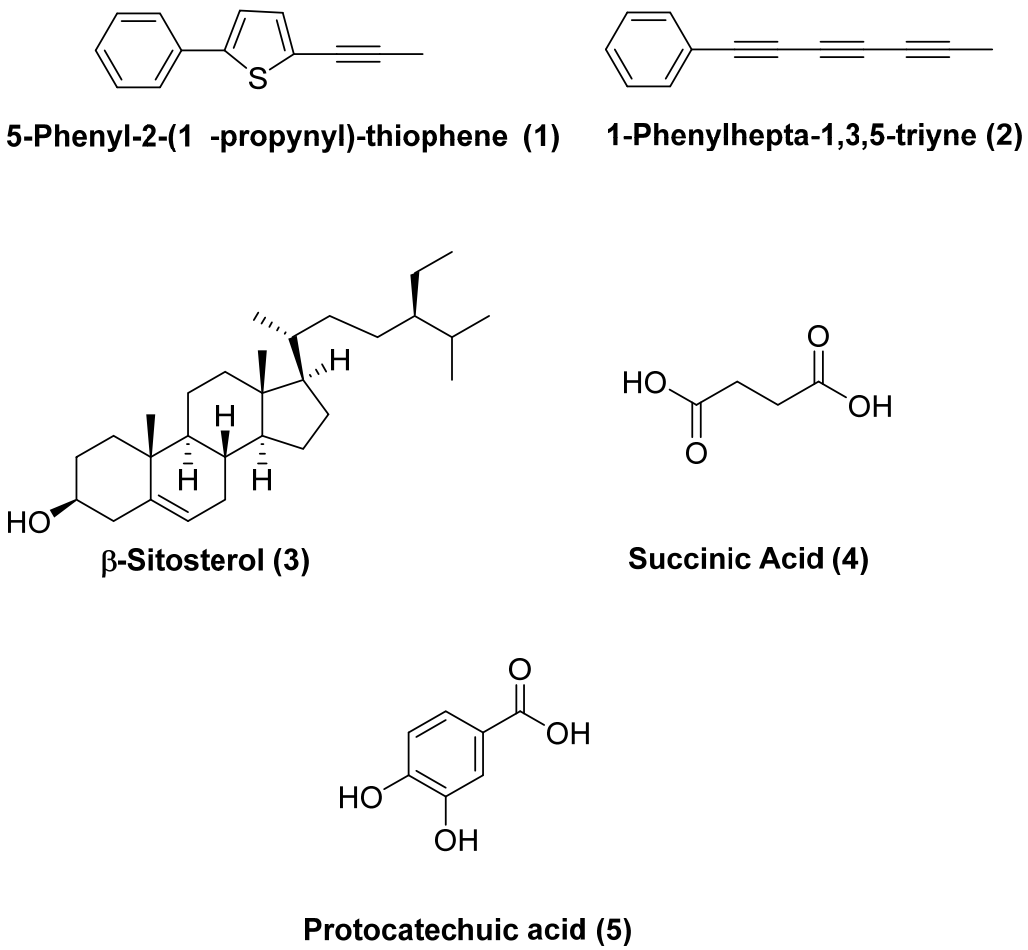


Figure 54. Chemical structure of isolated compounds from *Coreopsis lanceolata* stems.

4.3.2. Antitermite activity of isolated compounds from Coreopsis lanceolata stems

The antitermite effects against *C. curvignathus* were tested by no-choice test using paper discs impregnated with each isolated compound at a concentration of 1% for 14 days. Results of antitermite responses are summarized in **Figures 55 and 56**. Treatment of 5-phenyl-2-(1-propynyl)-thiophene (**1**) and 1-phenylhepta-1,3,5-triyne (**2**) greatly affected mortality and feeding of 33 termites.

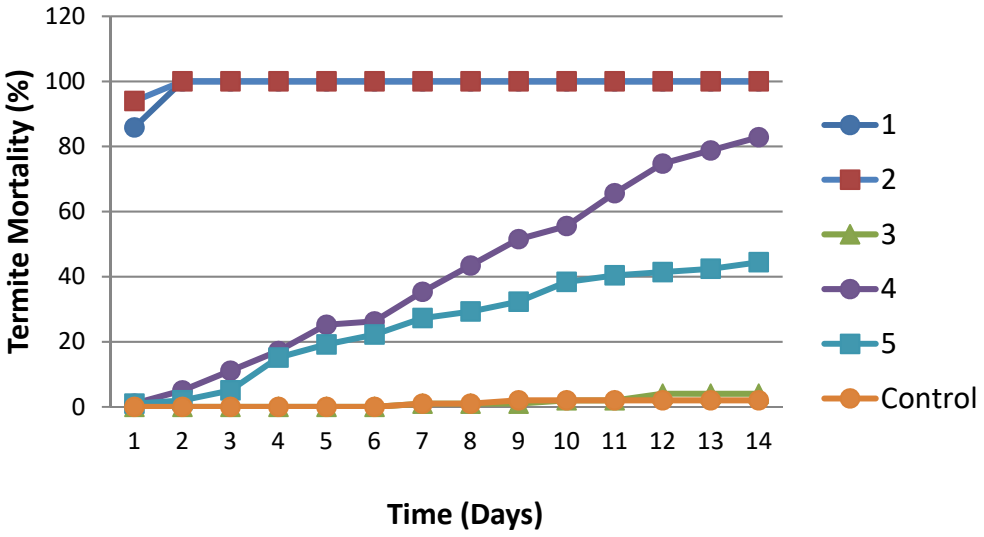


Figure 55. Termite mortality by treatment of isolated compounds from *Coreopsis lanceolata* stems against *Coptotermes curvignathus,* dose 1%, (Means, n = 3). 5-Phenyl-2-(1-propynyl)-thiophene (**1**), 1-phenylhepta-1,3,5-triyne (**2**), β-sitosterol (**3**), succinic acid (**4**) and protocatechuic acid (**5**).

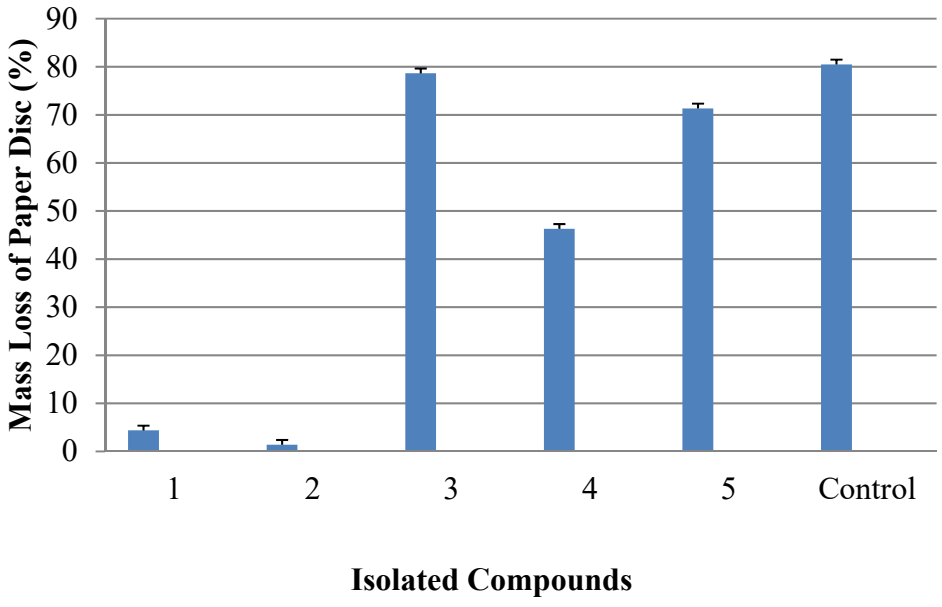


Figure 56. Paper disc consumption by *Coptotermes curvignathus* after 14 days exposure to isolated compounds from *Coreopsis lanceolata* stems. 5-Phenyl-2-(1-propynyl)-thiophene (**1**), 1-phenylhepta-1,3,5-triyne (**2**), β-sitosterol (**3**), succinic acid (**4**) and protocatechuic acid (**5**), dose 1%, (Means ± SEMs, n = 3).

The antitermite activities of compounds **1** and **2** were also evaluated in two doses of 0.5% and 0.25% again. (**Figures 57 and 58**). The dose-dependent effects of compounds **1** and **2** were observed. Even when sample doses were 0.5% and 0.25%, compounds **1** and **2** exerted quite high termicidal activity against *C. curvignathus*. At a dose of 0.5% **1** and **2**, all termites were killed within 2 days. Results shown in **Figure 57** confirmed that the antitermite activity of 1-phenylhepta-1,3,5-triyne (**2**) was higher than that of 5-phenyl-2-(1-propynyl)-thiophene (**1**).

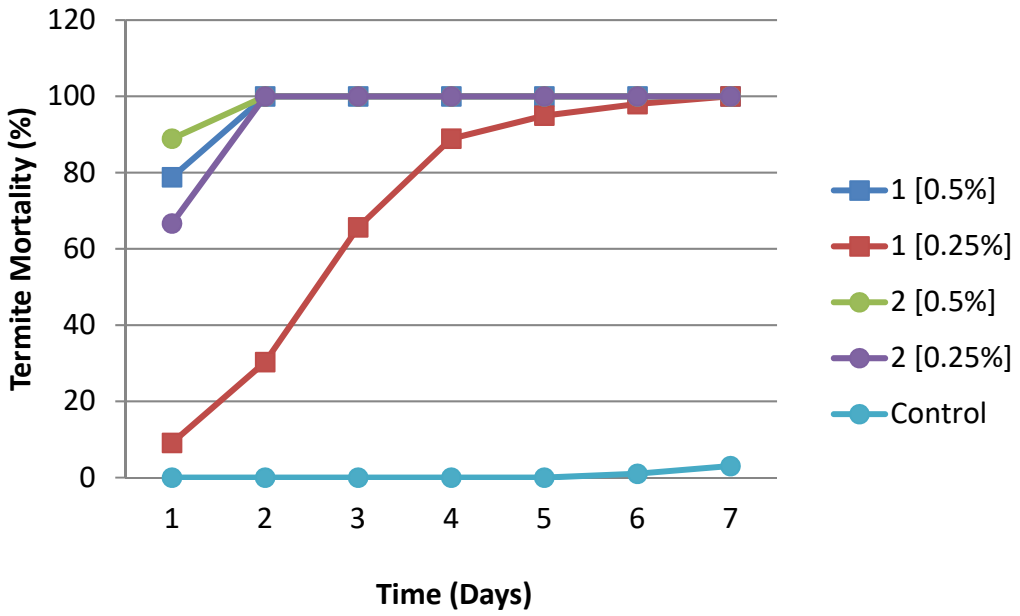


Figure 57. Termite mortality by treatment of 5-phenyl-2-(1-propynyl)-thiophene (**1**), 1-phenylhepta-1,3,5-triyne (**2**) isolated compounds from *Coreopsis lanceolata* stems against *Coptotermes curvignathus,* doses 0.5% and 0.25%, (Means, $n = 3$).

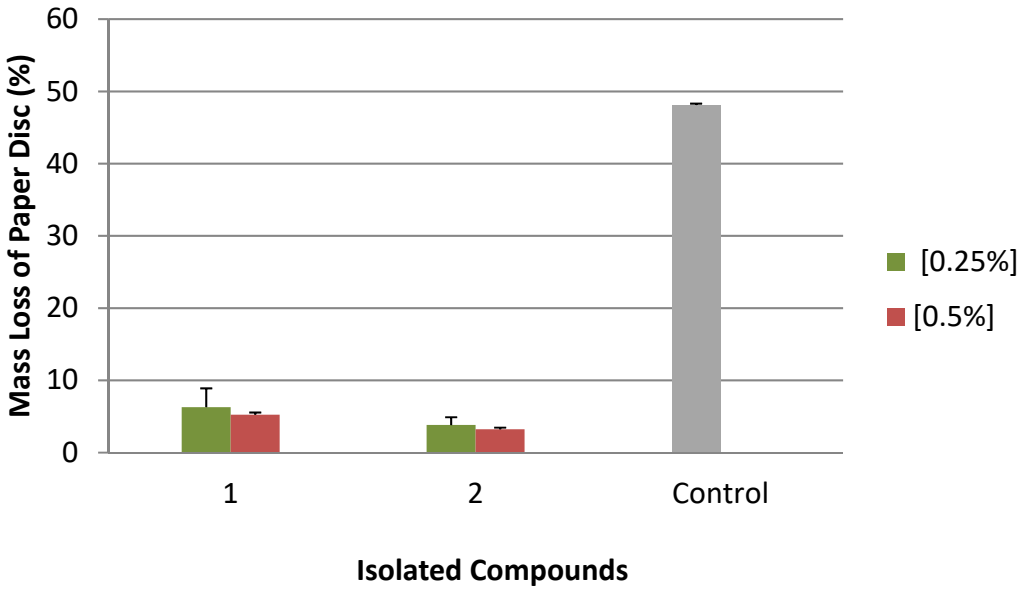


Figure 58. Paper disc consumption by *Coptotermes curvignathus* after 14 days exposure to 5-phenyl-2-(1-propynyl)-thiophene (**1**), 1-phenylhepta-1,3,5-triyne (**2**) isolated compounds from *Coreopsis lanceolata* stems. Doses 0.5% and 0.25%, (Means ± SEMs, $n = 3$).

These results implied that a thiophene and a triple bond existed in 5-phenyl-2-(1-propynyl)-thiophene (**1**), the triple bond conjugated to a benzene ring of 1-phenylhepta-1,3,5-triyne (**2**) play a significant role in their antitermite activity. Previously, Chang and Cheng described that the conjugated double bond such as cinnamaldehyde exhibited stronger antitermite activity (Chang and Cheng, 2002). Our data were similar to their results, we claim that the triple bond enhanced antitermite activity of compounds.

4.4. Conclusions

In conclusion, our result demonstrated that 5-phenyl-2-(1-propynyl)-thiophene (**1**) and 1-phenylhepta-1,3,5-triyne (**2**) in *n*-Hex fraction exerted strong potent antitermite activity against *C. curvignathus.* Our result will broaden the possibility of the application of *C. lanceolata* stems as an alternative for termite control.

References

Adfa, M., Kusnanda, A. J., Livandri, F., Rahmad, R., Darwis, W., Efdi, M., Ninomiya, M., Koketsu, M. 2017. Insecticidal activity of *Toona sinensis* against *Coptotermes curvignathus* Holmgren. *Rasayan J. Chem. 10*:153-159.

Azizuddin., Makhmoor, T., Choudary, M. I. 2010. Radical scavenging potential of compounds isolated from *Vitex agnus-castus. Turk. J. Chem. 34*:119-126.

Chan, S. P., Bong, C. F. J., Lau, W. H. 2011. Damage pattern and nesting characteristic of *Coptotermes curvignathus* (Isoptera: Rhinotermitidae) in oil palm on peat. *Am. J. Applied Sci. 8*:420-427.

Chang, S. T., Cheng, S. S. 2002. Antitermite activity of leaf essential oils and compounds from *Cinnamomum osmophleum*. *J. Agric. Food Chem. 50*:1389-1392.

Chaturvedula, V. S. P., Prakash, I. 2012. Isolation of stigmasterol and β-sitosterol from the dichloromethane extract of *Rubus suavissimus*. *Int. Curr. Pharm. J. 1*: 239-242.

El-attar, E., Galala, A., Gohar, A., Badria, F. 2015. Cycloartane glycoside: a new lactate dehydrogenase inhibitor, from the aerial part of agriculture waste of watermelon. *World J. Pharm. Sci. 3*:689-695.

Hiramatsu, Y., Adfa, M., Ninomiya, M., Yoshimura,T., Koketsu, M. 2013. Effects of 2,2-dimethylchromenesagainst the feeding behavior of *Coptotermes formosanus* Shiraki. *J. Pestic. Sci. 38*:228-231.

Kamboj, A., Saluja, A. K. 2011. Isolation of stigmasterol and β-sitosterol from petroleum ether extract of aerial parts of *Ageratum conyzoides* (Asteraceae). *Int. J. Pharm. Sci. 3*:94-96.

Kimura, Y., Hiraoka, K., Kawano, T., Fujioka, S., Shimada, A., 2008. Nematicidal activities of acetylene compounds from *Coreopsis lanceolata* L. *Z. Naturforsch 63c*:843-847.

Mishra, T., Srivastava, M., Kumar, A., Pal, M., Tewari, S. K. 2017. Chemical composition and termiticidal activity of artemisia nilagirica essential oil growing in Southern Hilly regions of India. *J. Essent. Oil Bear Pl.* DOI: 10.1080/0972060X.2016.1256235.

Ninomiya, M., Aoki, T., Adfa, M., Yoshimura,T., Koketsu, M. 2014. Comparison of antitermite properties of 2-thioxocoumarins against *Coptotermes formosanus* Shiraki. *Holzforschung, 68*:361-365.

Okada, Y., Okita, M., Murai, Y., Okano, Y., Nomura, M., 2014. Isolation an identification of flavonoids from *Coreopsis lanceolata* L. petals. *Nat. Prod. Res. 28:*201-204.

Oramahi, H. S., Yoshimura, T., 2014. Antifungal and antitermitic activities of wood vinegar from *Vitex pubescens* Vahl. *J. Wood Sci. 59*:344–350.

Pardede, A., Koketsu, M. 2017. Antioxidant and antileukemic activity of chemical components from bark of *Mangifera casturi*. *Comp. Clin. Pathol. 26*:499-504.

Pardede, A., Mashita, K., Ninomiya, M., Tanaka, K., Koketsu, M. 2016. Flavonoid profile and antileukemic activity of *Coreopsis lanceolata* flowers. *Bioorg. Med. Chem. Lett. 26*:2784-2787.

Raje, K. R., Hughes, G. P., Gondhalekar, A. D., Ginzel, M. D., Scharf, M. E. 2015. Toxicity of turmeric extracts to the termite Reticulitermes flavipes (Blattodea: Rhinotermitidae). *J. Econ. Entomol. 108*: 1480-1484.

Shang, Y. F., Oidovsambuu, S., Jeon, J. S., Nho, C. W., Um, B. H. 2013. Chalcones from the flowers of *Coreopsis lanceolata* and their in vitro antioxidative activity. *Planta. Med. 79*:295-300.

Sowmya, SN., Calistus, J. A. L., Deepa, M. 2016. Anti-termite properties of four selected species of Zingiberaceae rhizome extracts. *J. Zool. St. 3*:24-29.

Tanimoto, S., Miyazawa, M., Inoue, T., Okada, Y., Nomura, M. 2009. Chemical constituents of *Coreopsis lanceolata* L. and their physiological activities. *J. Oleo. Sci. 58*:141-146.

Acknowledgements

Alhamdulillah wasyukurillah, finally this thesis is finished. First and foremost I praise and acknowledge Allah SWT, the most beneficent and the most merciful. My humblest gratitude to the Holy Prophet Muhammad (Peace be upon him) whose way of life has been a continuous guidance for me. I thank to Allah SWT, for the way Allah SWT has upheld me and sustained me the throughout the study period. I thank for Allah SWT for giving me strength, health and for being gracious.

In this great opportunity, I wish to express my profound gratitude to my academic advisor Prof. Mamoru Koketsu for his patience, motivation, enthusiasm, immense knowledge and assistance throughout my years in his lab and also my daily life, best supervision and supporting me during my present study in his laboratory. His guidance helped me in all the time of research and writing of this thesis. He gave me an excellent preparation for becoming an autonomous research scientist. I am confident that the approach to scientific investigation I have cultivated under Prof. Mamoru Koketsu will serve me well in my future scientific endeavors.

I would like to thank Prof. Kaori Tanaka is acknowledged for helpful and hearty guidance in antileukemic, antioxidant and hepatoprotective assay system. I would also express my gratitude to Assist. Prof. Taro Udagawa, Assoc. Prof. Masaru Ishida and Assoc. Prof. Yuji Naruse for their support and fruitful cooperation to me.

I express my sincere thank to Prof. Toyohide Takeuchi and Prof. Funabiki Kazumasa, for the evaluation of the thesis and for their valuable comments and advices.

I should not forget to thank Assoc. Prof. Morina Adfa, who introduced me to Prof. Mamoru Koketsu and has always being my mentor during my scientific development and spirit to complete the doctoral program largely inspired by her. I would like to thank Prof. Yunazar Manjang, Assoc. Prof. Mai Efdi, Assoc. Prof. Agus Martono and Assist. Prof. Devi Ratnawati, my academic advisor during master and bachelor degree.

I greatly appreaciates the important assistance from Ph.D candidate Masayuki Ninomiya M.Sc expertise in structure elucidation of isolated compounds, UPLC-ESITOFMS, synthesis and biological activities. He was an extremely reliable source of practical knowledge, exceptionally intelligent and always full of new ideas. He constantly gave me valuable suggestion and kept me abreast with the state of the science that greatly boosted my research progress.

I would like to thank all personnel of the laboratory for nice working atmosphere in the laboratory and for endless friendship, love and unyielding support during my study period.

I would like to give my very special and gratitude to my parents, who have been supporting me in each and every step and breath I take, to my late father, whose love, kindness and spirit will always be in my heart and to my mother who makes me strength through her never-ended good wishes, pray and emotional support. I also would like to thank brothers and sisters for their support during all these years and prayer for me. Their support and believe in me throughout all

these years are the most driving forces for me to persue my dreams. Without them, this thesis would not exist.

I am thankful to Materials Engineering Division, Graduate School of Engineering, Gifu University and Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan for financial support to carry out the work described in this thesis and for awarding me the monbukagakusho scholarship.

To all members of PPI (Indonesian Student Society in Gifu, Japan), I thank you for your moral support, for many helps and kind friendship. Specials thank to Sri Mulyati M.Sc., Nur Nadia Binti Jasni M.Eng., Radhia Putri M.Sc., Rahma Yanda M.Eng., dr Winda Nestamer., Ali Rahmat M.AppSc., Okviyoandra Akhyar M.Sc., Taupik Rahman B.Sc., Fitry Yoni Yuliza M.Sc., Arif Juliari Kusnanda B.Sc., Angga Sanjaya B.Sc., Shota Fukuno M.Eng., Ryuta Inagaki M.Eng., Akinori Kakumu M.Eng., Komei Kato M.Eng., Shuko Kakuda M.Eng., Tsubasa Yonezawa M.Eng., Koharu Mashita B.Sc., Hironobu Irino M.Eng., Kenji Suzuki M.Eng., Nagumo Mina B.Sc., Sugimoto Hideya B.Sc and Rizaldi M.Sc for many helps, kind friendship and great moments that we shared together.

Finally, I am very sorry that I am not able to mention all people who have given a contribution to this thesis. I am sure that some people will be missing to be mentioned. I would like to thank all of you.

Thank you very much

Doumo Arigatou gozaimashita

Terima Kasih banyak

Antoni Pardede

Curriculum Vitae

Antoni Pardede was born on 29 July 1988 in Tangga Rasa, regency of empat lawang of South Sumatera Province, Indonesia. He finished the Elementary School (SDN 31), Junior High School (MTsN 1) and Senior High School (SMAN 3) in Bengkulu city. He received a grant from Directorate General of Higher Education of Republic Indonesia (The PPA scholarship) program for his Bachelor degree and obtained his B.Sc. degree in 2010 from Chemistry Department, Mathematics and Natural Sciences Faculty, Bengkulu University. Further, he obtained his master degree (M.Sc) from Chemistry Department, Mathematics and Natural Sciences Faculty, Andalas University, Padang, West Sumatera in 2012. After completing his M.Sc, he joined at Chemistry Education Department, Faculty of Teacher Training and Education, Islamic University of Kalimantan as a lecturer and researcher. He started his Ph.D study in April 2015 at Graduate School of Engineering, Gifu University with the research topic *Isolation of secondary metabolites from medicinal plants and invasive alien species and their biological activities* under supervisor Prof. Mamoru Koketsu. His Ph.D study was financially supported by Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan, (Monbukagakusho scholarship). During his study in Gifu University, he got a chance to attend scientific meeting to present his results and publish his research output as listed below.

List of Publications

1. **Antoni Pardede**, Mamoru Koketsu. 2017. Antioxidant and antileukemic activity of chemical components from bark of *Mangifera casturi*. *Comparative Clinical Pathology* ***26***(3): 499-504. https://link.springer.com/article/10.1007/s00580-016-2387-x

2. **Antoni Pardede**, Morina Adfa, Arif Juliari Kusnanda, Masayuki Ninomiya, Mamoru Koketsu. 2017. Flavonoid rutinosides from *Cinnamomum parthenoxylon* leaves and their hepatoprotective and antioxidant activity. *Medicinal Chemistry Research* ***26***(9): 2074-2079. https://link.springer.com/article/10.1007/s00044-017-1916-8

3. **Antoni Pardede**, Morina Adfa, Arif Juliari Kusnanda, Masayuki Ninomiya, Mamoru Koketsu. 2017. Isolation of secondary metabolites from *Stenochlaena palustris* stems and structure-activity relationships of 20-hydroxyecdysone derivatives on antitermite activity. Submitted to *Holzforschung*.

4. **Antoni Pardede**, Morina Adfa, Arif Juliari Kusnanda, Masayuki Ninomiya, Mamoru Koketsu. 2017. Chemical constituents of *Coreopsis lanceolata* stems and their antitermite activity against *Coptotermes curvignathus*. Accepted in *Journal of Economic Entomology.*

Following publications are not included in this thesis

1. **Antoni Pardede**, Koharu Mashita, Masayuki Ninomiya, Kaori Tanaka, Koketsu Mamoru. 2016. Flavonoid profile and antileukemic activity of *Coreopsis lanceolata* flowers. *Bioorganic & Medicinal Chemistry Letters* ***26*****(**12): 2784-2787. http://www.sciencedirect.com/science/article/pii/S0960894X16304450

2. Mai Efdi, **Antoni Pardede**, Akinori Kakumu, Hirokazu Hara, Syafrizayanti, Dessy Arisanti, Masayuki Ninomiya, Mamoru Koketsu. 2017. Chemical constituents of *Aglaia odorata* leaves and their anti-inflammatory effects. *Natural Product Communications* ***12***(11): 1717-1720. http://www.naturalproduct.us/JournalArchive.asp

3. Gideon Ampoma Gyebi, Joseph Oluwatope Adebayo, Olufunke Esan Olorundare, **Antoni Pardede**, Masayuki Ninomiya, Afolabi Olanrewaju Saheed, Abiola Samuel Babatunde, Mamoru Koketsu. 2017. Iloneoside: A cytotoxic ditigloylated pregnane glycoside from the leaves of *Gongronema latifolium* Benth. *Natural Product Research* in press. http://www.tandfonline.com/doi/full/10.1080/14786419.2017.1385019

List of Presentations

1. **Antoni Pardede** and Mamoru Koketsu. Antioxidant and antileukemic activity of chemical components from bark of *Mangifera casturi.* Joint Symposium Gifu University and Chonnam National University. *Oral presentation*. 1 February 2017. Gifu, Japan.

2. **Antoni Pardede**, Morina Adfa, Arif Juliari Kusnanda, Masayuki Ninomiya, Mamoru Koketsu. Secondary metabolites from *Cinnamomum parthenoxylon* leaves and their biological activities. 5th International Conference on Asian Network for Natural and Unnatural Materials (ANNUM V). *Oral presentation*. 11-12 July 2017. Jogjakarta, Indonesia.