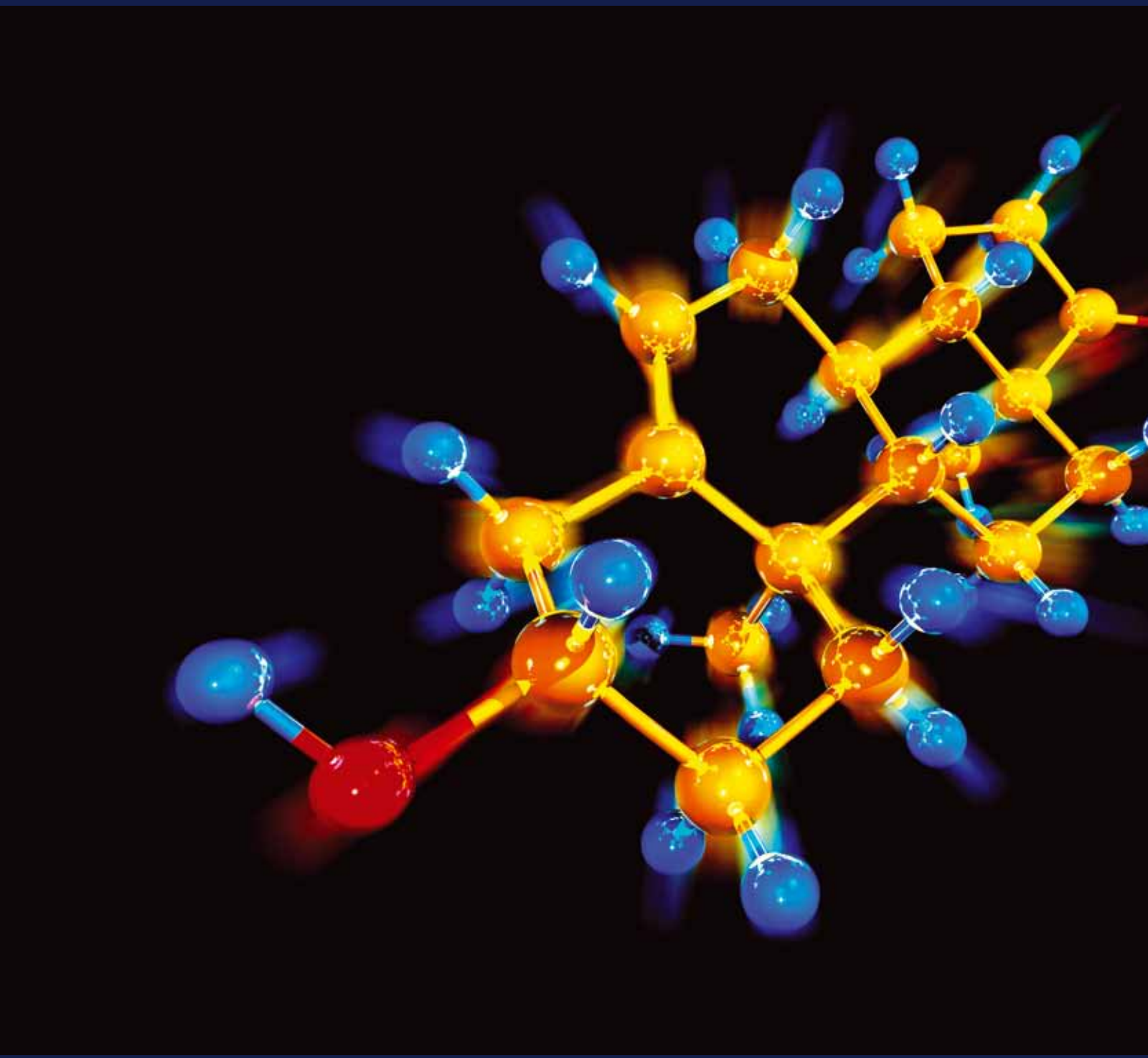
Journal of Chemistry

Bioactive Natural Products

Guest Editors: A. Hamid A. Hadi, Mehmet Emin Duru, and Ana B. Martin-Diana



Bioactive Natural Products

Journal of Chemistry

Bioactive Natural Products

Guest Editors: A. Hamid A. Hadi, Mehmet Emin Duru, and Ana B. Martin-Diana

Contents

Hindawi Publishing Corporation
Journal of Chemistry
Volume 2013, Article ID 208507, 1 page
http://dx.doi.org/10.1155/2013/208507

Editorial
Bioactive Natural Products

A. Hamid A. Hadi,[1] Mehmet Emin Duru,[2] and Ana B. Martin-Diana[3]

[1] *Department of Chemistry, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia*
[2] *Department of Chemistry, Faculty of Arts and Sciences, Mugla University, 48187 Mugla, Turkey*
[3] *School of Food and Environmental Health, Cathal Brugha Street, Dublin Institute of Technology, Dublin 2, Ireland*

Correspondence should be addressed to A. Hamid A. Hadi; ahamid@um.edu.my

Received 22 May 2013; Accepted 22 May 2013

Bioactive natural products have never ceased to play an important role in the search of novel therapeutic agents. Natural products from the nature such as the plant and marine sources form an integral part of the living system, and they have existed ever since the beginning of life. Therefore, it is undeniable that works related to natural products continue to develop in many aspects involving researchers from various scientific backgrounds. This special issue is dedicated to compile twelve articles on chemical and biological aspects of research developments in natural products.

Traditional medicinal plants still play a significant role in health maintenance and well-being that contribute to the economic growth. This is well described by R. A. Street et al. in his review on the commercially traditional medicinal plants of South Africa. The potential nutritional value of *Ilex paraguariensis* fruits, a native tree from Northeastern Argentina, as source of novel foods and medicine, is discussed by Laura Cogoi et al.

The article by K. Yokota et al. explains the application of ultraperformance liquid chromatography tandem mass spectrometry (UPLC) to determine the proportions of terminal and extension units of A-type and B-type highly polymeric proanthocyanidins. A. Giorgi et al. report on the analysis of volatile organic compounds from a Brazilian flora by Headspace Solid-Phase Microextraction coupled with Gas Chromatography and Mass Spectrometry (HS-SPME GC/MS) and its antioxidant property and repellent activity against *Aedes aegypti* L. The paper by J. Li et al. discussed the optimum conditions for the extraction of natural pigment from purple sweet potato as an alternative natural source of food coloring.

The inhibitory activity of plant and marine sources in cancer research are discussed in two papers. Z.-C. Kang et al. demonstrated the inhibitory effects of the aqueous extract from guava twigs on mutation and oxidative damage, and S. N. Fedorov et al. described the inhibitory effects of marine and algae extracts, thus supporting the role of natural products in the management of cancer.

The role of natural products in the treatment of Alzheimer's disease has yet to be fully exploited. The study by T. Zhao et al. demonstrated the potential inhibitory activity against acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) of β-carboline and quinoline alkaloids from the plants of genus *Peganum*. X. Ding et al. showed that the flavonoids from the leaves of *Ginkgo biloba*, a traditional Chinese medicinal plant, were found to inhibit AChE by enhancing acetylcholine levels and its potential insecticidal activity against brown planthopper.

Natural product synthesis also contributes to the development of new therapeutic agents. T. S. Zhivotova et al. illustrated the synthesis of 2,5-bi-substituted derivatives of 1,3,4-tiadiazol-2,5-ditiol and some of their biological properties. A. Rusina et al. report on the synthesis of complex c-glycosides of genistein and their antiproliferative activities.

Recent advances in molecular docking have enabled researchers to investigate the inhibitory activity of bioactive compounds. This is described by L. Hai-Boi et al. on the mechanism of selective inhibition of yohimbine and its derivatives on adrenoceptor $\alpha 2$ subtypes.

A. Hamid A. Hadi
Mehmet Emin Duru
Ana B. Martin-Diana

Hindawi Publishing Corporation
Journal of Chemistry
Volume 2013, Article ID 951392, 14 pages
http://dx.doi.org/10.1155/2013/951392

Research Article

C-Glycosidic Genistein Conjugates and Their Antiproliferative Activity

Aleksandra Rusin,[1] Maciej Chrubasik,[2] Katarzyna Papaj,[2] Grzegorz Grynkiewicz,[3] and Wiesław Szeja[2]

[1] *Center for Translational Research and Molecular Biology of Cancer, Maria Sklodowska-Curie Memorial Cancer Center and Institute of Oncology, Wybrzeze AK 15, 44-100 Gliwice, Poland*
[2] *Department of Organic Chemistry, Bioorganic Chemistry and Biotechnology, Silesian University of Technology, Krzywoustego 4, 44-100 Gliwice, Poland*
[3] *Pharmaceutical Research Institute, Rydygiera 8, 01-793 Warsaw, Poland*

Correspondence should be addressed to Wiesław Szeja; wieslaw.szeja@adres.pl

Received 25 October 2012; Revised 22 March 2013; Accepted 2 April 2013

Academic Editor: Mehmet Emin Duru

This paper presents our attempt to investigate scopes and the limitations of olefin cross-metathesis (CM) reaction in the synthesis of complex C-glycosides of genistein and evaluation of their antiproliferative activities. Novel genistein glycoconjugates were synthesized with the utility of CM reaction initiated by first and second generation of Grubbs catalysts. The relative reactivity of utilized olefins, based on categories proposed by Grubbs, was estimated. *In vitro* experiments in cancer cell lines showed that the selected derivatives (**3a** and **3f**) exhibited higher antiproliferative potential than the parent compound, genistein, and were able to block the cell cycle in the G2/M phase. The observed mechanism of action of C-glycosidic derivatives was similar to the activity of their O-glycosidic counterparts. These compounds were stable in culture medium. The obtained results show that our approach to genistein modification with application of cross-metathesis reaction allowed to obtain stable glycoconjugates with improved anticancer potential, compared to the parent isoflavone.

1. Introduction

The natural isoflavone, genistein abundant in soy-derived food and therefore a significant nonnutritional component of a diet is well known for at least two types of biological activity important for human health: hormonal (affinity to estrogen receptor beta) [1] and modulatory, influencing intracellular signaling and gene expression [2]. Epidemiological clinical and *in vitro* studies have shown that genistein might be a candidate for new drug in treatment of cardiovascular diseases, postmenopausal syndromes, and cancer [3–6]. Thus far, exploitation of these therapeutically prospective activities have not gone beyond early clinical trials, apparently because of rather poor drug-like properties of genistein (low lipophilicity combined with very low water solubility) reflected in pharmacokinetic parameters which practically exclude attaining of desired therapeutic concentrations of the active principle in the blood serum. It is postulated, however, that rational chemical derivatization of genistein can improve both pharmacokinetics and pharmacodynamics, including selectivity and efficacy of its biological action, and such claims have found experimental support in numerous studies [7]. Several groups have synthesized various derivatives of genistein with the intention to either improve biochemical and pharmacokinetic characteristics or to obtain compounds containing essential elements of the parent substance but having novel properties and/or affecting novel molecular targets [8–14]. In particular, research conducted by Polkowski and coworkers on O-glycosides of genistein should be mentioned [15, 16]. *In vitro* studies have shown that those derivatives are able to inhibit proliferation of various cancer cell lines. In a follow-up study, it has been found that glycoconjugates linking genistein with an unsaturated pyranoside through an alkyl chain show antiproliferative activity against cancer

cell lines, corroborating the working hypothesis, that an unsaturated sugar moiety is a carrier of certain structural features reflected in antiproliferative activity of genistein conjugates [17].

Pursuing further investigation of biological activity of genistein glycoconjugates, we decided to examine another type of constructs, less susceptible to biodegradation. Since O-glycosides can be easily hydrolyzed by enzymes present in the human body, we have focused on attachment of a sugar moiety via a C-glycosidic bond, reasoning that their greater stability will improve the bioavailability without affecting the antiproliferative activity. Although C-glycosylated flavonoids occur in nature, no practical synthetic alternative to the biogenetic pathway has been developed thus far. Therefore we decided to create a linkage between a carbohydrate moiety and a genistein molecule by an olefin cross-metathesis (CM) reaction with the easily accessible appropriate terminal olefins, that is, C-allyl glycosides and 7-O-allylgenistein.

Herein we present the synthesis of novel genistein glycoconjugates by application of olefin CM reaction initiated by first and second generation of Grubbs catalysts, their biological activity against selected tumor cell lines and stability in culture media.

2. Experimental

2.1. General Methods of Synthesis. Reactions were monitored by TLC, which was carried out on 0.25 mm Silica Gel F254 plates (Acros) using either UV light, a 5% EtOH solution of H_2SO_4 with heat as developing agent or a chamber with silica gel saturated with I_2. Acros Silica gel was used for column chromatography. ^{1}H and ^{13}C-NMR spectra were recorded on a VARIAN INOVA 300 (300 MHz and 75 MHz, resp.) and on VARIAN 600 instrument (600 MHz and 150 MHz, resp.) using Me_4Si as an internal reference.

2.2. General Procedure for Preparation of Grubbs Catalyst in Paraffin. Grubbs complex and paraffin were placed in a vessel sealed with septa. Then, air was removed by reduced pressure, and Ar gas was introduced. The vessel was placed in an ultrasonic H_2O bath and warmed to about 60°C which resulted in melting of the paraffin and homogenization of catalyst in it. After cooling the mixture, a brown wax was obtained, which was stored at r.t. without inert gas protection. The catalyst prepared in that way was still active after a 6-month period. The average concentration of Grubbs complex in paraffin was about 0.09 mmol/g.

2.3. General Procedure for Olefin Cross-Metathesis Reaction. 7-O-Allylgenistein (**2**) and C-allylglycoside (**1a**–**1h**) were dissolved in CH_2Cl_2 (solubility of **2** is about 20 mg per 1 mL). Grubb's catalyst in paraffin was placed inside a reflux condenser and then the condenser, was attached to the reaction vessel. Air was removed under reduced pressure (mixture was allowed to boil for about 2 min), and Ar gas was introduced. The mixture was heated at refluxed on a H_2O bath. The reaction started when the catalyst was washed from the condenser by the refluxing solvent. After 3 h, homodimer was removed from reaction mixture by filtration through a cotton wool, and the solvent was removed from the filtrate under reduced pressure. The residue was dissolved in toluene and chromatographed on silica-gel column.

2.4. Spectroscopic Data of Products Obtained in Olefin Cross-Metathesis Reactions. NMR samples of metathesis products contained both E and Z isomers. Analysis was done with the help of 2D NMR spectra: ^{1}H-^{1}H cosy and ^{1}H-^{13}C hetcor.

*2.4.1. 1″,4″-Bis-(5,4′-dihydroxyisoflavonyl-7-oxy)-but-2″-en (**5**)*

E Isomer. ^{1}H NMR (300 MHz, D_6-DMSO) δ ppm: 12.96 (**HO-5**, s 2H) 9.64 (**HO-4′**, s, 2H), 8.40 (**H-2**; s; 2H) 7.36 (**H-2′**, **H-6′**; d; $J_{11,15\text{-}12,14}$ = 8.6 Hz; 4H) 6.80 (**H-3′**, **H-5′**; d; $J_{12,14\text{-}11,15}$ = 8.6 Hz; 4H) 6.67 (**H-8**; d; $J_{5\text{-}7}$ = 2.2 Hz; 2H) 6.42 (**H-6**; d; $J_{7\text{-}5}$ = 2.2 Hz; 2H) 6.10 (**H-2″**, **H-3″**; m; 2H) 4.75 (**H-1″**, **H-4″**; m; 4H).

Z Isomer. ^{1}H NMR (300 MHz, d_6-DMSO) δ ppm: 12.93 (**H-5**; s; 2H) 9.64 (**H-4′**; s; 2H) 8.38 (**H-2**; s; 2H) 7.36 (**H-2′**, **H-6′**; d; $J_{11,15\text{-}12,14}$ = 8.6 Hz; 4H) 6.80 (**H-3′**, **H-5′**; d; $J_{12,14\text{-}11,15}$ = 8.6 Hz; 4H) 6.69 (**H-8**; d; $J_{5\text{-}7}$ = 2.2 Hz; 2H) 6.45 (**H-6**; d; $J_{7\text{-}5}$ = 2.2 Hz; 2H) 5.93 (**H-2″**, **H-3″**; m; 2H) 4.87 (**H-1″**, **H-4″**; m; 4H).

Due to extremely low solubility of compound **5** we were unable to record ^{13}C NMR and HRMS spectra.

*2.4.2. 7′-O-(1‴-C-(2,3,4,6-Tetra-O-acetyl-α-D-glucopyranosyl)-but-2‴-en-4‴-yl)-genistein (**3a**)*

E Isomer. ^{1}H NMR (600 MHz, DMSO-D6) δ ppm: 12.94 (**OH-5′**; s; 1H) 9.60 (**OH-4″**; s; 1H) 8.39 (**H-2′**; s; 1H) 7.39 (**H-2″**, **H-6″**; d; $J_{11'\text{-}12',15'\text{-}14'}$ = 8.8 Hz; 2H) 6.83 (**H-3″**, **H-5″**; d; $J_{12'\text{-}11',14'\text{-}15'}$ = 8.8 Hz; 2H) 6.65 (**H-8′**; d; $J_{5'\text{-}7'}$ = 2.4 Hz; 1H) 6.40 (**H-6′**; d; $J_{7'\text{-}5'}$ = 2.4 Hz; 1H) 5.87 (**H-3‴**; dt; $J_{3''\text{-}2''}$ = 14.6 Hz; $J_{3''\text{-}4''}$ = 5.4 × 2 Hz; 1H) 5.81 (**H-2‴**; ddd; $J_{2''\text{-}3''}$ = 14.6 Hz; $J_{2''\text{-}1L''}$ = 6.8 Hz; $J_{2''\text{-}1''a}$ = 6.3 Hz; 1H) 5.30 (**H-3**; dd; $J_{3\text{-}2}$ = 8.8 Hz; $J_{3\text{-}4}$ = 8.8 Hz; 1H) 4.91 (**H-2**; dd; $J_{2\text{-}3}$ = 8.8 Hz; $J_{2\text{-}1}$ = 5.9 Hz; 1H) 4.86 (**H-4**; dd; $J_{4\text{-}3}$ = 9.1 Hz; $J_{4\text{-}5}$ = 9.1 Hz; 1H) 4.64 (**H-4″**; d; $J_{4''\text{-}3''}$ = 5.4 Hz; 2H) 4.21–4.13 (**H-1**, **H-6a**; m; 2H) 4.00–3.93 (**H-5**, **H-6b**; m; 2H) 2.71 (**H-1‴a**; m; $J_{1''a\text{-}1''b}$ = 15.0 Hz; $J_{1''a\text{-}2''}$ = 10.9 Hz; $J_{1''a\text{-}1}$ = 7.6 Hz; 1H) 2.32 (**H-1‴b**; m; 1H) 2.03; 2.00; 1.99; 1.98 (**CH$_3$**–; 12H).

^{13}C NMR (150 MHz, DMSO-D6) δ ppm: 180.37 (**C-4′**) 170-169 (**C=O** × **4**, **C-6′**) 164.12 (**C-5′**) 161.74 (**C-4″**) 157.49 (**C-8a′**) 154.28 (**C-2′**) 130.67 (**C-2‴**) 130.13 (**C-2″**, **C-6″**) 126.79 (**C-3‴**) 122.51 (**C-3′**) 121.04 (**C-1″**) 115.08 (**C-3″**, **C-5″**) 105.42 (**C-4′a**) 98.49 (**C-8′**) 92.96 (**C-6′**) 71.13 (**C-1**) 69.81 (**C-2**) 69.46 (**C-3**) 68.38 (**C-4**, **C-5**) 68.79 (**C-4‴**) 61.80 (**C-6**) 28.42 (**C-1‴**) 20.45 (**CH$_3$**– × 4).

Z Isomer. ^{1}H NMR (600 MHz, DMSO-D6) δ ppm 12.96 (**OH-5′**; s; 1H) 9.60 (**OH-4″**; s; 1H; overlapped) 8.40 (**H-2′**; s; 1H) 7.39 (**H-2″**, **H-6″**; 2H; overlapped) 6.83 (**H-3″**, **H-5″**; 2H; overlapped) 6.69 (**H-8′**; d; $J_{5'\text{-}7'}$ = 2.35 Hz; 1H) 6.45 (**H-6′**; d; $J_{7'\text{-}5'}$ = 2.35 Hz; 1H) 5.76 (**H-3‴**; dt; $J_{3''\text{-}2''}$ = 10.50 Hz; $J_{3''\text{-}4''}$ = 5.40 × 2 Hz; 1H) 5.63 (**H-2‴**; ddd; $J_{2''\text{-}3''}$ = 10.50 Hz;

$J_{2''\text{-}1''b}$ = 6.80 Hz; $J_{2''\text{-}1''a}$ = 6.30 Hz; 1H) 5.37 (**H-3**; dd; $J_{3\text{-}2}$ = 9.10 Hz; $J_{3\text{-}4}$ = 9.10 Hz; 1H) 4.94 (**H-2**; dd; $J_{2\text{-}3}$ = 9.10 Hz; $J_{2\text{-}1}$ = 5.87 Hz; 1H) 4.86 (**H-4**; 1H; overlapped) 4.80 (**H-4‴**; d; $J_{4''\text{-}3''}$ = 5.40 Hz; 2H) 4.21–4.13 (**H-1**, **H-6a**; m; 2H) 4.00/3.93 (**H-5**, **H-6b**; m; 2H) 2.82 (**H-1‴a**; m; $J_{1''a\text{-}1''b}$ = 15.26 Hz; $J_{1''a\text{-}2''}$ = 9.39 Hz; $J_{1''a\text{-}1}$ = 9.39 Hz; 1H) 2.38 (**H-1‴b**; m; 1H) 2.02; 2.01; 1.99; 1.98 (**CH**$_3$–; 12H).

^{13}C NMR (150 MHz, DMSO-D6) δ ppm: 180.37 (**C-4′**; overlapped) 170-169 (**C=O** × 4, **C-6′**) 164.23 (**C-5′**) 161.74 (**C-4″**; overlapped) 157.42 (**C-8′a**) 154.28 (**C-2′**; overlapped) 130.13 (**C-2″**, **C-6″**; overlapped) 129.98 (**C-2‴**) 126.00 (**C-3‴**) 122.51 (**C-3′**; overlapped) 121.04 (**C-1″**; overlapped) 115.08 (**C-3″**, **C-5″**; overlapped) 105.42 (**C-4′a**; overlapped) 98.49 (**C-8′**; overlapped) 93.12 (**C-6′**) 71.55 (**C-1**) 69.90 (**C-2**) 69.46 (**C-3**; overlapped) 68.38 (**C-4, C-5**; overlapped) 64.89 (**C-4‴**) 61.80 (**C-6**; overlapped) 24.58 (**C-1‴**) 20.45 (**CH**$_3$ × 4; overlapped).

HRMS [M + Na]$^+$. Experimental: 677.1821; calculated: 677.1841.

2.4.3. 1′,4′-Bis-C-(2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl)-but-2′-en (4a)

E Isomer. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 5.50 (**H-2′**, **H-3′**; t; 3.8 Hz × 2; 2H) 5.31 (**H-3**; dd; $J_{3\text{-}2}$ = 9.3 Hz; $J_{3\text{-}4}$ = 8.9 Hz; 2H) 5.07 (**H-2**; dd; $J_{2\text{-}3}$ = 9.3 Hz; $J_{2\text{-}1}$ = 5.4 Hz; 2H) 5.00 (**H-4**; dd; $J_{4\text{-}3}$ = 8.9 Hz; $J_{4\text{-}5}$ = 8.9 Hz; 2H) 4.26 (**H-6a**; dd; $J_{6a\text{-}6b}$ = 12.2 Hz; $J_{6a\text{-}5}$ = 5.0 Hz; 2H) 4.21 (**H-1**; ddd; $J_{1\text{-}1'a}$ = 10.5 Hz, $J_{1\text{-}2}$ = 5.4 Hz; $J_{1\text{-}1'b}$ = 4.9 Hz; 2H) 4.06 (**H-6b**; dd; $J_{6b\text{-}6a}$ = 12.2 Hz; $J_{6b\text{-}5}$ = 2.9 Hz; 2H) 3.85 (**H-5**; ddd; $J_{5\text{-}4}$ = 8.9 Hz; $J_{5\text{-}6a}$ = 5.0 Hz; $J_{5\text{-}6b}$ = 2.9 Hz; 2H) 2.52 (**H-1′a**, **H-4′a;** ddd; $J_{1'a\text{-}1'b}$ = 15.0 Hz; $J_{1'a\text{-}1}$ = 10.5 Hz; J = 4.0 Hz; 2H) 2.28 (**H-1′b**, **H-4′b**; ddd; $J_{1'b\text{-}1'a}$ = 15.0 Hz; $J_{1'b\text{-}1}$ = 4.9 Hz; J = 4.0 Hz; 2H) 2.09 (**OAc**; s; 6H) 2.06 (**OAc**; s; 6H) 2.04 (**OAc**; s; 12H).

^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 170.62 (**C=O**) 170.08 (**C=O**) 169.57 (**C=O**) 169.46 (**C=O**) 127.82 (**C-2′**, **C-3′**) 72.06 (**C-1**) 70.19 (**C-2**) 70.06 (**C-3**) 68.87 (**C-4**) 68.60 (**C-5**) 62.07 (**C-6**) 29.33 (**C-1′**, **C-4′**) 20.69/20.63 (**CH**$_3$).

Z Isomer. ^{1}H NMR (600 MHz, $CDCl_3$) δ ppm: 5.52 (**H-2′**, **H-3′**; t; 4.4 Hz × 2; 2H).

^{13}C NMR (150 MHz, $CDCl_3$) δ ppm: 170.40 (**C=O**) 170.03 (**C=O**) 169.63 (**C=O**) 69.53 (**C=O**) 126.52 (**C-2′**, **C-3′**) 72.06 (**C-1**, overlapped) 70.19 (**C-2,** overlapped) 70.06 (**C-3**, overlapped) 69.17 (**C-4**) 68.70 (**C-5**) 61.99 (**C-6**) 24.56 (**C-1′**, **C-4′**) 20.69/20.63 (**CH**$_3$).

HRMS [M + Na]$^+$. Experimental: 739.2404; calculated: 739.2420.

2.4.4. 7′-O-(1‴-C-(2,3,4,6-Tetra-O-acetyl-α-D-galactopyranosyl)-but-2‴-en-4‴-yl)-genistein (3b)

E Isomer. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 12.81 (**H-5′**; s; 1H) 7.84 (**H-2′**; s; 1H) 7.36 (**H-2″**, **H-6″**; d; $J_{11',15'\text{-}12',14'}$ = 8.6 Hz; 2H) 6.87 (**H-3″**, **H-5″**; d; $J_{12',14'\text{-}11',15'}$ = 8.6 Hz; 2H) 6.37 (**H-8′**; d; $J_{5'\text{-}7'}$ = 2.2 Hz; 1H) 6.34 (**H-6′**; d; $J_{7'\text{-}5'}$ = 2.2 Hz; 1H) 5.82/5.78 (**H-2‴**, **H-3‴**; m; 2H) 5.44 (**H-4**; dd; $J_{4\text{-}5}$ = $J_{4\text{-}3}$ = 2.4 Hz; 1H) 5.30/5.21 (**H-2**, **H-3**; m; 2H) 4.52 (**H-4‴**; d; $J_{4''\text{-}3''}$ = 3.7 Hz; 2H) 4.36–4.28 (**H-1**, **H-6a**; m; 2H) 4.13–4.05 (**H-5**, **H-6b**; m; 2H) 2.61–2.28 (**H-1‴a**, **H-1‴b**; m; 2H) 2.13 (**CH**$_3$; s; 3H) 2.10 (**CH**$_3$; s; 3H) 2.06 (**CH**$_3$; s; 3H) 2.05 (**CH**$_3$; s; 3H).

^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 20.63 (**CH**$_3$) 20.72 (**CH**$_3$ × 2) 20.76 (**CH**$_3$) 29.65 (**C-1‴**) 61.20 (**C-6**) 67.37 (**C-5**) 67.85 (**C-4‴**) 68.26 (**C-3**) 68.49 (**C-4**) 68.70 (**C-2**) 71.19 (**C-1**) 92.99 (**C-8′**) 98.69 (**C-6′**) 106.24 (**C-4′a**) 115.59 (**C-3″**, **C-5″**) 122.58 (**C-3′**) 123.66 (**C-1″**) 126.97 (**C-3‴**) 130.16 (**C-2‴**) 130.24 (**C-2″**, **C-6″**) 152.76 (**C-2′**) 156.28 (**C-4″**) 157.84 (**C-8′a**) 162.56 (**C-5′**) 164.36 (**C-7′**) 169.92 (**C=O**) 170.06 (**C=O**) 170.14 (**C=O**) 170.72 (**C=O**) 180.86 (**C-4′**).

Z Isomer. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 12.81 (**H-5′**; overlapped; 1H) 7.85 (**H-2′**; s; 1H) 7.36 (**H-2″**, **H-6″**; overlapped; 2H) 6.87 (**H-3″**, **H-5″**; overlapped; 2H) 6.41 (**H-8′**; d; $J_{5'\text{-}7'}$ = 2.2 Hz; 1H) 6.37 (**H-6′**; overlapped; 1H) 5.87–5.78 (**H-3‴**; m; 1H) 5.71 (**H-2‴**; dt; $J_{2''\text{-}3''}$ = 11.4; $J_{2''\text{-}1''}$ = 7.5 × 2 Hz; 1H) 5.44 (**H-4**; overlapped; 1H) 5.30–5.21 (**H-2**, **H-3**; overlapped; 2H) 4.62 (**H-4‴**; d; $J_{4''\text{-}3''}$ = 5.9 Hz; 2H) 4.36–4.28 (**H-1**, **H-6a**; overlapped; 2H) 4.13–4.05 (**H-5**, **H-6b**; overlapped; 2H) 2.61–2.28 (**H-1‴a**, **H-1‴b**; overlapped; 2H) 2.13-2.05 (**CH**$_3$; overlapped; 12H).

^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 20.63/20.76 (**CH**$_3$) 25.55 (**C-1‴**) 61.06 (**C-6**) 64.43 (**C-4‴**) 67.25 (**C-5**) 67.85 (**C-3**) 68.43 (**C-4**) 68.83 (**C-2**) 71.19 (**C-1**; overlapped) 92.99 (**C-8′**; overlapped) 98.72 (**C-6′**) 106.30 (**C-4′a**) 115.59 (**C-3″**, **C-5″**; overlapped) 122.58 (**C-3′**; overlapped) 123.69 (**C-1″**) 126.25 (**C-3‴**) 129.47 (**C-2‴**) 130.24 (**C-2″**, **C-6″**; overlapped) 152.76 (**C-2′**; overlapped) 156.28 (**C-4″**; overlapped) 157.87 (**C-8′a**) 162.59 (**C-5′**) 164.36 (**C-7′**; overlapped) 169.92 (**C=O**; overlapped) 170.00 (**C=O**) 170.10 (**C=O**) 170.76 (**C=O**) 180.86 (**C-4′**; overlapped).

HRMS [M + Na]$^+$. Experimental: 677.1819; calculated: 677.1841.

2.4.5. 1′,4′-Bis-C-(2,3,4,6-tetra-O-acetyl-α-D-galactopyranosyl)-but-2′-en (4b)

E Isomer. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 5.48 (**H-2′**, **H-3′**; t; 3.7 Hz × 2; 2H) 5.41 (**H-4**; dd; $J_{4\text{-}3}$ = $J_{4\text{-}5}$ = 2.7 Hz; 2H) 5.26/5.22 (**H-2**; m; 2H) 5.19 (**H-3**; dd; $J_{3\text{-}4}$ = 2.7; $J_{3\text{-}2}$ = 9.1 Hz; 2H) 4.34/4.19 (**H-1**, **H-6a**; m; 4H) 4.11/4.02 (**H-5**, **H-6b**; m; 4H) 2.48/2.38 (**H-1′a**, **H-4′a**; m; 2H) 2.28/2.18 (**H-1′b**, **H-4′b**; m; 4H) 2.12 (**CH**$_3$; s; 6H) 2.08 (**CH**$_3$; s; 6H) 2.06 (**CH**$_3$; s; 6H) 2.04 (**CH**$_3$; s; 6H).

^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 20.65 (**CH**$_3$) 20.73 (**CH**$_3$) 20.77 (**CH**$_3$) 29.67 (**C-1′**, **C-4′**) 60.98 (**C-6**) 67.28 (**C-5**) 67.83 (**C-3**) 68.23 (**C-2**) 68.43 (**C-4**) 71.38 (**C-1**) 128.03 (**C-2′**, **C-3′**) 169.75 (**C=O**) 169.89 (**C=O**) 170.05 (**C=O**) 170.51 (**C=O**).

Z Isomer. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 5.51 (**H-2′**, **H-3′**; t; 4.4 Hz × 2). Only **H-2′** and **H-3′** signals of Z isomer were not overlapped by signals of isomer E.

^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 20.65/20.77 (**CH**$_3$) 24.81 (**C-1′**, **C-4′**) 61.13 (**C-6**) 67.36 (**C-5**) 67.83 (**C-3**) 68.50

(**C-2**) 68.64 (**C-4**) 71.38 (**C-1**) 126.77 (**C-2′**, **C-3′**) 169.85 (**C=O** × 2) 170.02 (**C=O**) 170.59 (**C=O**).

HRMS [M + Na]$^+$. Experimental: 739.2416; calculated: 739.2420.

*2.4.6. 7′-O-(1‴-C-(2,3,4,6-Tetra-O-acetyl-α-D-mannopyranosyl)-but-2‴-en-4‴-yl)-genistein (**3c**)*

E Isomer. ^{1}H NMR (600 MHz, d_6-DMSO) δ ppm: 12.91 (**H-5′**; s; 1H) 9.62 (**H-4″**; s; 1H) 8.34 (**H-2′**; s; 1H) 7.37 (**H-2″**, **H-6″**; dd; $J_{11'15'\text{-}12',14'}$ = 8.6 Hz; $J_{11'\text{-}15'}$ = 2.2 Hz; 2H) 6.81 (**H-3″**, **H-5″**; dd; $J_{12'14'\text{-}11',15'}$ = 8.5 Hz; $J_{12'\text{-}14'}$ = 2.2 Hz; 2H) 6.60 (**H-8′**; d; $J_{5'\text{-}7'}$ = 2.2 Hz; 1H) 6.36 (**H-6′**; d; $J_{7'\text{-}5'}$ = 2.2 Hz; 1H) 5.84 (**H-3‴**; dt; $J_{3''\text{-}2''}$ = 15.5 Hz; $J_{3''\text{-}4''}$ = 5.4 × 2 Hz; 1H) 5.79 (**H-2‴**; ddd; $J_{2''\text{-}3''}$ = 15.5 Hz; $J_{2''\text{-}1''a}$ = 6.4 Hz; $J_{2''\text{-}1''b}$ = 6.4 Hz; 1H) 5.24 (**H-3**; dd; $J_{3\text{-}4}$ = 9.0 Hz; $J_{3\text{-}2}$ = 3.3 Hz; 1H) 5.07 (**H-2**; dd; $J_{2\text{-}1}$ = 3.3 Hz; $J_{2\text{-}3}$ = 3.3 Hz; 1H) 5.02 (**H-4**; dd; $J_{4\text{-}5}$ = $J_{4\text{-}3}$ = 8.5 Hz; 1H) 4.61 (**H-4‴**; d; $J_{4''\text{-}3''}$ = 5.4 Hz; 2H) 4.20 (**H-6a**; dd; $J_{6a\text{-}6b}$ = 12.2 Hz; $J_{6a\text{-}5}$ = 6. Hz; 1H) 3.99/3.93 (**H-1**, **H-5**, **H-6b**; m; 3H) 2.69/2.59 (**H-1‴a**; m; 1H) 2.43 (**H-1‴b**; ddd; $J_{1''b\text{-}1''a}$ = 14.6 Hz; $J_{1''b\text{-}2''}$ = 6.4 Hz; $J_{1''b\text{-}1}$ = 5.8 Hz; 1H).

^{13}C NMR (150 MHz, d_6-DMSO) δ ppm: 20.46 (**CH$_3$**) 20.49 (**CH$_3$**) 20.54 (**CH$_3$**) 20.68 (**CH$_3$**) 31.26 (**C-1‴**) 61.90 (**C-6**) 64.90 (**C-4‴**) 66.66 (**C-4**) 68.30 (**C-3**) 69.79 (**C-2**) 69.83 (**C-5**) 73.37 (**C-1**) 93.01 (**C-8′**) 98.55 (**C-6′**) 105.48 (**C-4′a**) 115.14 (**C-3″**, **C-5″**) 121.10 (**C-3′**) 122.56 (**C-1″**) 127.09 (**C-3‴**) 130.17 (**C-2″**, **C-6″**, **C-2‴**) 154.28 (**C-2′**) 157.50 (**C-4″**, **C-8′a**) 161.75 (**C-5′**) 164.15 (**C-7′**) 169.56 (**C=O** × 2) 169.77 (**C=O**) 170.08 (**C=O**) 180.41 (**C-4′**).

Z Isomer. ^{1}H NMR (600 MHz, $CDCl_3$) δ ppm: 12.82 (**H-5′**; s; 1H) 7.85 (**H-2′**; s; 1H) 7.39 (**H-2″**, **H-6″**; d; $J_{11',15'\text{-}12',14'}$ = 8.7 Hz; 2H) 6.89 (**H-3″**, **H-5″**; d; $J_{11',15'\text{-}12',14'}$ = 8.7 Hz; 2H) 6.43 (**H-8′**; d; $J_{5'\text{-}7'}$ = 2.4 Hz; 1H) 6.40 (**H-6′**; d; $J_{7'\text{-}5'}$ = 2.4 Hz; 1H) 5.86 (**H-3‴**; ddd; $J_{3''\text{-}2''}$ = 11.0 Hz; $J_{3''\text{-}4''a}$ = $J_{3''\text{-}4''b}$ = 6.3 Hz; $J_{3''\text{-}1''a}$ = $J_{3''\text{-}1''b}$ = −1.4 Hz; 1H) 5.73 (**H-2‴**; ddd; $J_{2''\text{-}3''}$ = 11.0 Hz; $J_{2''\text{-}1''a}$ = $J_{2''\text{-}1''b}$ = 7.3; $J_{2''\text{-}4''a}$ = $J_{2''\text{-}4''b}$ = −1.3 Hz; 1H) 5.28 (**H-3**; dd; $J_{3\text{-}4}$ = 8.2 Hz; $J_{3\text{-}2}$ = 3.4 Hz; 1H) 5.20 (**H-2**; dd; $J_{2\text{-}1}$ = 4.1 Hz; $J_{2\text{-}3}$ = 3.4 Hz; 1H) 5.18 (**H-4**; dd; $J_{4\text{-}3}$ = 8.2 Hz; $J_{4\text{-}5}$ = 7.6 Hz; 1H) 4.68 (**H-4‴a**; ddd; $J_{4''a\text{-}4''b}$ = 12.3 Hz; $J_{4''a\text{-}3''}$ = 6.3 Hz; $J_{4''a\text{-}2''}$ = −1.3 Hz; 1H) 4.65 (**H-4‴b**; ddd; $J_{4''b\text{-}4''a}$ = 12.3 Hz; $J_{4''b\text{-}3''}$ = 6.3 Hz; $J_{4''b\text{-}2''}$ = −1.3 Hz; 1H); 4.42 (**H-6a**; dd; $J_{6a\text{-}6b}$ = 12.1 Hz; $J_{6a\text{-}5}$ = 6.7 Hz; 1H) 4.12 (**H-6b**; dd; $J_{6b\text{-}6a}$ = 12.1 Hz; $J_{6b\text{-}5}$ = 3.3 Hz; 1H) 4.06 (**H-1**; ddd; $J_{1\text{-}1''a}$ = 8.7; $J_{1\text{-}1''b}$ = 5.9 Hz; $J_{1\text{-}2}$ = 4.1 Hz; 1H) 2.58 (**H-1‴a**; dddd; $J_{1''a\text{-}1''b}$ = 15.0 Hz; $J_{1''a\text{-}1}$ = 8.7 Hz; $J_{1''a\text{-}2''}$ = 7.3 Hz; $J_{1''a\text{-}3''}$ = −1.4 Hz; 1H) 2.53 (**H-1‴b**; dddd; $J_{1''a\text{-}1''b}$ = 15.0 Hz; $J_{1''a\text{-}1}$ = 5.9; $J_{1''a\text{-}2''}$ = 7.3 Hz; $J_{1''a\text{-}3''}$ = −1.4 Hz; 1H) 2.12 (**CH$_3$**; s; 3H) 2.09 (**CH$_3$**; s; 6H) 2.06 (**CH$_3$**; s; 3H).

^{13}C NMR (150 MHz, $CDCl_3$) δ ppm: 20.74 (**CH$_3$**) 20.78 (**CH$_3$**) 20.81 (**CH$_3$**) 20.93 (**CH$_3$**) 28.23 (**C-1‴**) 62.05 (**C-6**) 64.50 (**C-4‴**) 67.13 (**C-4**) 68.59 (**C-3**) 69.72 (**C-2**) 71.33 (**C-5**) 73.33 (**C-1**) 93.10 (**C-8′**) 98.81 (**C-6′**) 106.39 (**C-4′a**) 115.60 (**C-3″**, **C-5″**) 122.95 (**C-3′**) 123.70 (**C-1″**) 126.94 (**C-3‴**) 128.69 (**C-2‴**) 130.32 (**C-2″**, **C-6″**) 152.74 (**C-2′**) 156.04 (**C-4″**) 157.92 (**C-8′a**) 162.73 (**C-5′**) 164.43 (**C-7′**) 169.70 (**C=O**) 169.96 (**C=O**) 170.21 (**C=O**) 170.72 (**C=O**) 180.86 (**C-4′**).

HRMS [M + Na]$^+$. Experimental: 677.1805; calculated: 677.1841.

*2.4.7. 1′,4′-Bis-C-(2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl)-but-2′-en (**4c**)*. Signals of Z isomer were completely overlapped by signals of isomer E.

E Isomer. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 5.57 (**H-2′**, **H-3′**; t; 3.3 Hz × 2; 2H) 5.27/5.17 (**H-2**, **H-3**, **H-4**; m; 6H) 4.34 (**H-6a**; dd; $J_{6a\text{-}6b}$ = 12.0 Hz; $J_{6a\text{-}5}$ = 5.8 Hz; 2H) 4.10 (**H-6b**; dd; $J_{6b\text{-}6a}$ = 12.0 Hz; $J_{6b\text{-}5}$ = 3.0 Hz; 2H) 4.00 (**H-1**; ddd; $J_{1\text{-}1'a}$ = 7.2 Hz; $J_{1\text{-}1'a}$ = 7. Hz; $J_{1\text{-}1'a}$ = 2.9 Hz; 2H) 3.9 (**H-5**; ddd; $J_{5\text{-}4}$ = 6.3 Hz; $J_{5\text{-}6a}$ = 5.8 Hz; $J_{5\text{-}6b}$ = 3.0 Hz; 2H) 2.54/2.36 (**H-1′a**, **H-1′b**, **H-4′a**, **H-4′b**; m; 4H) 2.12 (**CH$_3$**; s; 6H) 2.10 (**CH$_3$**; s; 6H) 2.07 (**CH$_3$**; s; 6H) 2.03 (**CH$_3$**; s; 6H).

^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 20.94 (**CH$_3$**) 20.77 (**CH$_3$** × 2) 20.70 (**CH$_3$**) 32.50 (**C-1′**, **C-4′**) 62.34 (**C-6**) 66.93 (**C-4**) 68.69 (**C-3**) 69.82 (**C-2**) 70.72 (**C-5**) 74.29 (**C-1**) 128.00 (**C-2′**, **C-3′**) 169.64 (**C=O**) 169.95 (**C=O**) 170.14 (**C=O**) 170.66 (**C=O**).

HRMS [M + Na]$^+$. Experimental: 739.2433; calculated: 739.2420.

*2.4.8. 7′-O-(1‴-C-(2,3,4,6-Tetra-O-benzyl-α-D-glucopyranosyl)-but-2‴-en-4‴-yl)-genistein (**3d**)*

E Isomer. ^{1}H NMR (600 MHz, $CDCl_3$) δ ppm: 12.80 (**OH-5′**; s; 1H) 7.77 (**H-2′**; s; 1H) 7.33/7.11 (–**Ph**, **H-2″**, **H-6″**; m; 22H) 6.82 (**H-3″**, **H-5″**; d; $J_{12'\text{-}11',14'\text{-}15'}$ = 8.2 Hz; 2H) 6.32 (**H-8′**; d; $J_{5'\text{-}7'}$ = 2.4 Hz; 1H) 6.31 (**H-6′**; d; $J_{7'\text{-}5'}$ = 2.4 Hz; 1H) 5.83 (**H-2‴**; d; $J_{2''\text{-}3''}$ = 15.6; $J_{2''\text{-}1''a}$ = 7.0 Hz; $J_{2''\text{-}1''b}$ = 7.0 Hz; 1H) 5.68 (**H-3‴**; dt; $J_{3''\text{-}2''}$ = 15.6 Hz; $J_{3''\text{-}4''}$ = 5.9 Hz × 2; 1H) 4.94 (–**OCH$_2$Ph**; d; J_{gem} = 10.9; 1H) 4.82 (–**OCH$_2$Ph**; d; J_{gem} = 10.6; 1H) 4.81 (–**OCH$_2$Ph**; d; J_{gem} = 10.9 Hz; 1H) 4.70 (–**OCH$_2$Ph**; d; J_{gem} = 11.7 Hz; 1H) 4.61 (–**OCH$_2$Ph**; d; J_{gem} = 11.7 Hz; 1H) 4.59 (–**OCH$_2$Ph**; d; J_{gem} = 12.2 Hz; 1H) 4.47 (–**OCH$_2$Ph**; d; J_{gem} = 10.6 Hz; 1H) 4.46 (–**OCH$_2$Ph**; d; J_{gem} = 12.2 Hz; 1H) 4.40 (**H-4‴**; d; $J_{4''\text{-}3''}$ = 5.9 Hz; 2H) 4.13 (**H-1**; ddd; $J_{1\text{-}2}$ = 5.2; $J_{1\text{-}1''a}$ = 10.6 Hz; $J_{1\text{-}1''b}$ = 5.0 Hz; 1H) 3.81 (**H-3**; $J_{3\text{-}2}$ = 9.4 Hz; $J_{3\text{-}4}$ = 7.9 Hz; 1H) 3.76 (**H-2**; dd; $J_{2\text{-}3}$ = 9.4 Hz; $J_{2\text{-}1}$ = 5.7 Hz; 1H) 3.71–3.66 (**H-6a**; m; 1H) 3.65/3.59 (**H-4**, **H-5**, **H-6b**; m; 3H) 2.53 (**H-1‴a**, **H-1‴b**; m; 2H).

^{13}C NMR (150 MHz, $CDCl_3$) δ ppm: 180.79 (**C-4′**) 164.53 (**C-7′**) 162.48 (**C-8′a**) 157.81 (**C-5′**) 156.21 (**C-4″**) 152.65 (**C-2′**) 138.59 (**Ph**) 138.05 (**Ph**) 137.92 (**Ph**) 137.83 (**Ph**) 131.83 (**C-2‴**) 130.18 (**C-2″**, **C-6″**) 128.45/127.60 (**Ph** × 4) 126.19 (**C-3‴**) 123.58 (**C-3′**) 122.59 (**C-1″**) 115.62 (**C-3″**, **C-5″**) 106.14 (**C-4′a**) 98.82 (**C-6′**) 92.95 (**C-8′**) 82.18 (**C-3**) 79.90 (**C-2**) 78.07 (**C-4**) 75.43 (**CH$_2$Ph**) 75.13 (**CH$_2$Ph**) 73.52 (**C-1**) 73.43 (**CH$_2$Ph**)

73.16 (**C**H_2**Ph**) 71.18 (**C-5**) 68.93 (**C-6**) 68.97 (**C-4‴**) 28.43 (**C-1‴**).

Z Isomer. ^{1}H NMR (600 MHz, $CDCl_3$) δ ppm: 12.79 (**OH-5′**; s; 1H) 7.77 (**H-2′**; s; 1H; overlapped) 7.33/7.11 (–**Ph**, **H-2″**, **H-6″**; m; 22H; overlapped) 6.82 (**H-3″**, **H-5″**; 2H; overlapped) 6.35 (**H-8′**; d; $J_{5'\text{-}7'}$ = 2.35 Hz; 1H) 6.34 (**H-6′**; d; $J_{7'\text{-}5'}$ = 2.35 Hz; 1H) 5.77 (**H-2‴**, **H-3‴**; m; 2H) 4.93 (–**OCH**$_2$**Ph**; d; J_{gem} = 10.86 Hz; 1H) 4.82 (–**OCH**$_2$**Ph**; d; J_{gem} = 10.56 Hz; 1H) 4.81 (–**OCH**$_2$**Ph**; 1H; overlapped) 4.71 (–**OCH**$_2$**Ph**; d; J_{gem} = 11.44 Hz; 1H) 4.60 (–**OCH**$_2$**Ph**; d; J_{gem} = 11.74 Hz; 1H) 4.60 (–**OCH**$_2$**Ph**; d; J_{gem} = 12.03 Hz; 1H) 4.47 (–**OCH**$_2$**Ph**; 1H; overlapped) 4.46 (–**OCH**$_2$**Ph**; 1H; overlapped) 4.58 (**H-4‴**; 2H; overlapped) 4.13 (**H-1**; ddd; $J_{1\text{-}2}$ = 5.21; $J_{1\text{-}1''a}$ = 10.64 Hz; $J_{1\text{-}1''b}$ = 5.0 Hz; 1H) 3.81 (**H-3**; 1H; overlapped) 3.76 (**H-2**; 1H; overlapped) 3.71/3.66 (**H-6a**; 1H; overlapped) 3.65/3.59 (**H-4**, **H-5**, **H-6b**; 3H; overlapped) 2.53 (**H-1‴a**, **H-1‴b**; 2H; overlapped).

^{13}C NMR (150 MHz, $CDCl_3$) δ ppm: 180.83 (**C-4′**) 164.51 (**C-7′**) 162.55 (**C-8′a**) 157.84 (**C-5′**) 156.16 (**C-4″**) 152.68 (**C-2′**) 138.59–137.83 (**Ph** × 4; overlapped) 130.94 (**C-2‴**) 130.18 (**C-2″**, **C-6″**; overlapped) 128.45–127.60 (**Ph** × 4) 125.51 (**C-3‴**) 123.62 (**C-3′**) 122.63 (**C-1″**) 115.62 (**C-3″**, **C-5″**; overlapped) 106.23 (**C-4′a**) 98.83 (**C-6′**) 92.98 (**C-8′**) 82.12 (**C-3**) 79.82 (**C-2**) 77.91 (**C-4**) 75.40 (**CH$_2$Ph**) 75.05 (**CH$_2$Ph**) 73.89 (**C-1**) 73.48 (**CH$_2$Ph**) 73.32 (**CH$_2$Ph**) 71.55 (**C-5**) 68.87 (**C-6**) 64.59 (**C-4‴**) 24.14 (**C-1‴**).

2.4.9. 1′,4′-Bis-C-(2,3,4,6-tetra-O-benzylo-α-D-glukopyranosyl)-but-2′-en (4d)

E Isomer. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 7.09/7.34 (**Ph**; m; 40H) 5.51 (**H-2′**, **H-3′**; t; 3.5 Hz × 2; 2H) 4.94/4.41 (**CH$_2$Ph**; m; 16H) 4.07 (**H-1**; ddd; $J_{1\text{-}2}$ = 5.6 Hz; $J_{1\text{-}1'a}$ = 9.0 Hz; $J_{1\text{-}1'b}$ = 4.6 Hz; 2H) 3.84/3.54 (**H-2**, **H-3**, **H-4**, **H-5**, **H-6a**, **H-6b**; m; 12H) 2.50/2.41 (**H-1′a**, **H-1′b**, **H-4′a**, **H-4′b**; m; 4H).

Z Isomer. ^{1}H NMR (75 MHz, $CDCl_3$) δ ppm: 5.56 (**H-2′**, **H-3′**; t; 4.0 Hz × 2; 2H). Only H-2′ and H-3′ signals were not overlapped by signals of E isomer.

2.4.10. 7′-O-(1‴-C-(3,4,6-Tri-O-acetyl-2-deoxy-α-D-glucopyranosyl)-but-2‴-en-4‴-yl)-genistein (3e)

E Isomer. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 12.81 (**H-5′**; s; 1H) 7.83 (**H-2′**; s; 1H) 7.35 (**H-2″**, **H-6″**; d; $J_{11',15'\text{-}12',14'}$ = 8.5 Hz; 2H) 6.86 (**H-3″**, **H-5″**; d; $J_{12',14'\text{-}11',15'}$ = 8.5 Hz; 2H) 6.37 (**H-8′**; d; $J_{5'\text{-}7'}$ = 2.1 Hz; 1H) 6.34 (**H-6′**; d; $J_{7'\text{-}5'}$ = 2.1 Hz; 1H) 5.82 (**H-2‴**; dddt; $J_{2''\text{-}3''}$ = 15.6 Hz; $J_{2''\text{-}1''a}$ = $J_{2''\text{-}1''b}$ = 7.1 Hz; $J_{2''\text{-}4''}$ = –1.5 × 2; 1H) 5.79 (**H-3‴**; dtd; $J_{3''\text{-}2''}$ = 15.6 Hz; $J_{3''\text{-}4''}$ = 5.4 × 2; $J_{3''\text{-}1''a}$ = $J_{3''\text{-}1''b}$ = –1.5 Hz; 1H) 5.14 (**H-3**; ddd; $J_{3\text{-}2b}$ = 7.6 Hz; $J_{3\text{-}4}$ = 7.0 Hz; $J_{3\text{-}2a}$ = 4.6 Hz; 1H) 4.89 (**H-4**; dd; $J_{4\text{-}3}$ = $J_{4\text{-}5}$ = 7.0 Hz; 1H) 4.51 (**H-4‴**; d; $J_{4''\text{-}3''}$ = 3.7 Hz; 2H) 4.43 (**H-6a**; dd; $J_{6a\text{-}6b}$ = 12.1 Hz; $J_{6a\text{-}5}$ = 6.2 Hz; 1H) 4.12–4.06 (**H-1**, **H-6b**; m; 2H) 3.94 (**H-5**; ddd; $J_{5\text{-}4}$ = 7.0 Hz; $J_{5\text{-}6a}$ = 6.2 Hz; $J_{5\text{-}6b}$ = 3.4 Hz; 1H) 2.56 (**H-1‴a**; dddd; $J_{1''a\text{-}1''b}$ = –13.0 Hz; $J_{1''a\text{-}1}$ = 8.5; $J_{1''a\text{-}2''}$ = 7.1 Hz; $J_{1''a\text{-}2''}$ = –1.5 Hz; 1H) 2.34 (**H-1‴b**; dddd; $J_{1''b\text{-}1''a}$ = –13.0 Hz; $J_{1''b\text{-}1}$ = 5.2 Hz; $J_{1''b\text{-}2''}$ = 7.1 Hz; $J_{1''b\text{-}3''}$ = –1.5 Hz; 1H) 2.10 (**CH$_3$**; s; 3H) 2.08 (**CH$_3$**; s; 3H) 2.08 (**CH$_3$**; s; 3H) 2.00 (**H-2a**; ddd; $J_{2a\text{-}2b}$ = 14.0 Hz; $J_{2a\text{-}1}$ = 4.8 Hz; $J_{2a\text{-}3}$ = 4.6 Hz; 1H) 1.90–1.82 (**H-2b**; m; 1H).

^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 20.77 (**CH$_3$**) 20.80 (**CH$_3$**) 21.02 (**CH$_3$**) 31.97 (**C-2**) 35.32 (**C-1‴**) 61.91 (**C-6**) 68.36 (**C-4**) 68.56 (**C-3**) 68.72 (**C-4‴**) 69.42 (**C-1**) 70.91 (**C-5**) 93.04 (**C-8′**) 98.70 (**C-6′**) 106.19 (**C-4′a**) 115.59 (**C-3″**, **C-5″**) 122.47 (**C-3′**) 123.63 (**C-1″**) 126.85 (**C-3‴**) 130.63 (**C-2‴**) 130.20 (**C-2″**, **C-6″**) 152.74 (**C-2′**) 156.34 (**C-4″**) 157.81 (**C-8′a**) 162.51 (**C-5′**) 164.39 (**C-7′**) 169.57 (**C=O**) 170.22 (**C=O**) 170.95 (**C=O**) 180.85 (**C-4′**).

Z Isomer. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 12.81 (**H-5′**; s; 1H) 7.84 (**H-2′**; s; 1H) 7.35 (**H-2″**, **H-6″**; overlapped; 2H) 6.86 (**H-3″**, **H-5″**; overlapped; 2H) 6.42 (**H-8′**; d; $J_{5'\text{-}7'}$ = 2.1 Hz; 1H) 6.38 (**H-6′**; d; $J_{7'\text{-}5'}$ = 2.1 Hz; 1H) 5.85–5.79 (**H-3‴**; overlapped; 1H) 5.72 (**H-2‴**; ddd; $J_{2''\text{-}3''}$ = 10.9 Hz; $J_{2''\text{-}1''a}$ = $J_{2''\text{-}1''b}$ = 7.3 Hz; 1H) 5.14 (**H-3**; overlapped; 1H) 4.88 (**H-4**; dd; $J_{4\text{-}3}$ = $J_{4\text{-}5}$ = 6.6 Hz; 1H) 4.64 (**H-4‴**; d; $J_{4''\text{-}3''}$ = 5.8 Hz; 2H) 4.48 (**H-6a**; dd; $J_{6a\text{-}6b}$ = 11.9 Hz; $J_{6a\text{-}5}$ = 6.9 Hz; 1H) 4.12–4.06 (**H-1**, **H-6b**; m; 2H) 3.96 (**H-5**; partially overlapped; 1H) 2.60–2.51 (**H-1‴a**; overlapped; 1H) 2.40 (**H-1‴b**; dd; partially overlapped; $J_{1''b\text{-}2''}$ = 7.3; $J_{1''b\text{-}1}$ = 6.0 Hz; 1H) 2.10–2.08 (**CH$_3$**; zakryte; 9H) 2.00 (**H-2a**; overlapped; 1H) 1.90–1.82 (**H-2b**; overlapped; 1H).

^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 20.77 (**CH$_3$**; overlapped) 20.80 (**CH$_3$**; overlapped) 21.02 (**CH$_3$**; overlapped) 31.13 (**C-1‴**) 31.88 (**C-2**) 61.78 (**C-6**) 64.47 (**C-4‴**) 68.17 (**C-4**) 68.52 (**C-3**) 69.20 (**C-1**) 71.22 (**C-5**) 93.04 (**C-8′**; overlapped) 98.74 (**C-6′**) 106.23 (**C-4′a**) 115.59 (**C-3″**, **C-5″**; overlapped) 122.47 (**C-3′**; overlapped) 123.63 (**C-1″**; overlapped) 126.27 (**C-3‴**) 129.73 (**C-2‴**) 130.20 (**C-2″**, **C-6″**; overlapped) 152.74 (**C-2′**; overlapped) 156.34 (**C-4″**; overlapped) 157.84 (**C-8′a**) 162.51 (**C-5′**; overlapped) 164.42 (**C-7′**) 169.57 (**C=O**; overlapped) 170.22 (**C=O**; overlapped) 170.95 (**C=O**; overlapped) 180.85 (**C-4′**; overlapped).

HRMS [M + Na]$^+$. Experimental: 619.1772; calculated: 619.1786.

2.4.11. 1′,4′-Bis-C-(2,3,4,6-tetra-O-acetyl-2-deoxy-α-D-glucopyranosyl)-but-2′-en (4e)

E Isomer. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 5.57 (**H-2′**, **H-3′**; m; 2H) 5.11 (**H-3**; ddd; $J_{3\text{-}4}$ = 7.3 Hz; $J_{3\text{-}2a}$ = 4.5 Hz; $J_{3\text{-}2b}$ = 8.0 Hz; 2H) 4.88 (**H-4**; dd; $J_{4\text{-}5}$ = $J_{4\text{-}3}$ = 7.3 Hz; 2H) 4.39 (**H-6a**; dd; $J_{6a\text{-}6b}$ = 12.0 Hz; $J_{6a\text{-}5}$ = 5.9 Hz; 2H) 4.07 (**H-6b**; dd; $J_{6b\text{-}6a}$ = 12.0 Hz; $J_{6b\text{-}5}$ = 3.2 Hz; 2H) 4.07/3.98 (**H-1**; m; 2H) 3.89 (**H-5**; ddd; $J_{5\text{-}4}$ = 7.3; $J_{5\text{-}6a}$ = 5.9; $J_{5\text{-}6b}$ = 3.2 Hz; 2H) 2.51/2.22 (**H-1′a**, **H-1′b**, **H-4′a**, **H-4′b**; m; 4H) 2.09 (**CH$_3$**; s; 6H) 2.07 (**CH$_3$**; s; 6H) 2.06 (**CH$_3$**; s; 6H) 2.02/1.93 (**H-2a**; m; 2H) 1.90/1.79 (**H-2b**; m; 2H).

^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 20.80 (**CH$_3$**) 20.83 (**CH$_3$**) 21.04 (**CH$_3$**) 35.56 (**C-1′**, **C-4′**) 31.92 (**C-2**) 62.06 (**C-6**)

68.58 (**C-4**) 68.66 (**C-3**) 70.09 (**C-1**) 70.73 (**C-5**) 128.46 (**C-2′**, **C-3′**) 169.75 (**C=O**) 170.03 (**C=O**) 170.73 (**C=O**).

Z Isomer. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 5.57 (**H-2′**, **H-3′**; overlapped; 2H) 5.14 (**H-3**; partially overlapped; 2H) 4.87 (**H-4**; dd; $J_{4\text{-}5} = J_{4\text{-}3} = 7.0$ Hz; 2H) 4.42 (**H-6a**; dd; $J_{6a\text{-}6b} = 12.0$ Hz; $J_{6a\text{-}5} = 6.4$ Hz; 2H) 4.1/4.05 (**H-6b**; overlapped; 2H) 4.07/3.98 (**H-1**; overlapped; 2H) 3.92 (**H-5**; ddd; $J_{5\text{-}4} = 7.0$; $J_{5\text{-}6a} = 6.4$ Hz; $J_{5\text{-}6b} = 3.4$ Hz; 2H) 2.51/2.22 (**H-1′a**, **H-1′b**, **H-4′a**, **H-4′b**; partially overlapped; 4H) 2.09/2.06 ($\mathbf{CH_3}$; overlapped; 18H) 2.02/1.93 (**H-2a**; overlapped; 2H) 1.90/1.79 (**H-2b**; overlapped; 2H).

^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 20.80/21.04 ($\mathbf{CH_3}$) 30.56 (**C-1′**, **C-4′**) 32.00 (**C-2**) 61.98 (**C-6**) 68.47 (**C-4**) 68.66 (**C-3**) 69.84 (**C-1**) 70.99 (**C-5**) 127.10 (**C-2′**, **C-3′**) 169.75/170.73 (**C=O**).

HRMS [M + Na]$^+$. Experimental: 623.2328; calculated: 623.2310.

*2.4.12. 7′-O-(1‴-C-(4-O-Acetyl-2,3,6-trideoxy-α-L-erythro-heks-2-enpyranosyl)-but-2‴-en-4‴-yl)-genistein (**3f**)*

E Isomer. ^{1}H NMR (600 MHz, $CDCl_3$) δ ppm: 12.80 (**OH-5**; s; 1H) 7.82 (**H-2′**; s; 1H) 7.34 (**H-2″**, **H-6″**; d; $J_{10'\text{-}11',14'\text{-}13'} = 8.63$ Hz; 2H) 6.85 (**H-3″**, **H-5″**; d; $J_{11'\text{-}10',13'\text{-}14'} = 8.63$ Hz; 2H) 6.36 (**H-8′**; d; $J_{7'\text{-}5'} = 1.99$ Hz; 1H) 6.39 (**H-6′**; d; $J_{5'\text{-}7'} = 2.65$ Hz; 1H) 5.92 (**H-2‴**; ddd; $J_{2''\text{-}3''} = 15.1$; $J_{2''\text{-}1''a} = J_{2''\text{-}1''b} = 7.3$ Hz; 1H) 5.94–5.92 (**H-2**; m; 1H) 5.82 (**H-3**; ddd; $J_{3\text{-}2} = 10.1$ Hz; $J_{3\text{-}4} = 3.5$; $J_{3\text{-}1} = 1.7$ Hz; 1H) 5.79 (**H-3‴**; dt; $J_{3''\text{-}2''} = 15.1$ Hz; $J_{3''\text{-}4''} = 6.0$ Hz × 2; 1H) 4.91 (**H-4**; broad signal; 1H) 4.53 (**H-4‴**; d; $J_{4''\text{-}3''} = 6.0$ Hz; 2H) 4.26 (**H-1**; ddd; $J_{1\text{-}1''a} = J_{1\text{-}1''b} = 7.3$ Hz; $J_{1\text{-}2} = 1.99$ Hz; 1H) 3.96 (**H-5**; dq; $J_{5\text{-}6} = 6.63$ Hz × 3; $J_{5\text{-}4} = 4.64$ Hz; 1H) 2.49 (**H-1‴a**; ddd; $J_{1''a\text{-}1''b} = 14.60$ Hz; $J_{1''a\text{-}1} = J_{1''a\text{-}2''} = 7.30$ Hz; 1H) 2.38 (**H-1‴b**; ddd; $J_{1''b\text{-}1''a} = 14.60$ Hz; $J_{1''b\text{-}1} = J_{1''b\text{-}2''} = 7.30$ Hz; 1H) 2.10 (**OAc**; s; 3H) 1.26 (**H-6**; t; $J_{6\text{-}5} = 6.63$ Hz × 3; 3H).

^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 180.87 (**C-4′**) 170.07 (**C=O**) 164.46 (**C-7′**) 162.42 (**C-5′**) 157.81 (**C-4″**) 156.39 (**C-8′a**) 152.77 (**C-2′**) 33.20 (**C-2**) 131.38 (**C-2‴**) 130.15 (**C-2″**, **C-6″**) 126.60 (**C-3‴**) 123.65 (**C-3′**) 123.06 (**C-3**) 122.30 (**C-1″**) 115.64 (**C-3″**, **C-5″**) 106.14 (**C-4′a**) 98.78 (**C-8′**) 93.02 (**C-6′**) 69.53 (**C-1**) 69.36 (**C-4**) 68.88 (**C-5**, **C-4‴**) 36.74 (**C-1‴**) 21.19 ($\mathbf{CH_3}$–) 16.74 (**C-6**).

Z Isomer. ^{1}H NMR (600 MHz, $CDCl_3$) δ ppm: 12.81 (**OH-5′**; s; 1H) 7.84 (**H-2′**; s; 1H) 7.34 (**H-2″**, **H-6″**; overlapped; 2H) 6.85 (**H-3″**, **H-5″**; overlapped; 2H) 6.40 (**H-8′**; d; $J_{5'\text{-}7'} = 2.32$ Hz; 1H) 6.37 (**H-6′**; d; $J_{7'\text{-}5'} = 2.3$ Hz; 1H) 5.94 (**H-2**; ddd; $J_{2\text{-}3} = 10.1$ Hz; $J_{2\text{-}1} = 2.7$ Hz; $J_{2\text{-}4} = -1.3$ Hz) 5.85 (**H-3**; ddd; $J_{3\text{-}2} = 10.1$ Hz; $J_{3\text{-}4} = 3.5$ Hz; $J_{3\text{-}1} = -1.7$ Hz; 1H) 5.80/5.83 (**H-2‴**; **H-3‴**; overlapped; 2H) 4.91 (**H-4**; overlapped; 1H) 4.65 (**H-4‴**; d; $J_{4''\text{-}3''} = 4.6$ Hz; 2H) 4.26 (**H-1**; overlapped; 1H) 3.96 (**H-5**; overlapped; 1H) 2.49 (**H-1‴a**; overlapped; 1H) 2.44 (**H-1‴b**; ddd; $J_{1''b\text{-}1''a} = 14.60$ Hz; $J_{1''b\text{-}1} = J_{1''b\text{-}2''} = 6.0$ Hz; 1H) 2.09 (**OAc**; s; 3H) 1.27 (**H-6**; t; $J_{6\text{-}5} = 6.63$ Hz × 3; 3H).

^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 180.87 (**C-4′**; overlapped) 170.07 (**C=O**; overlapped) 164.46 (**C-7′**; overlapped) 162.46 (**C-5′**) 157.81 (**C-4″**; overlapped) 156.39 (**C-8′a**; overlapped) 152.77 (**C-2′**; overlapped) 133.09 (**C-2**) 130.15 (**C-2‴**, **C-2″**, **C-6″**; overlapped) 126.16 (**C-3‴**) 123.68 (**C-3′**) 123.23 (**C-3**) 122.30 (**C-1″**; overlapped) 115.64 (**C-3″**, **C-5″**; overlapped) 106.17 (**C-4′a**) 98.72 (**C-8′**) 93.02 (**C-6′**; overlapped) 69.31 (**C-1**) 69.27 (**C-4**) 69.07 (**C-5**) 64.54 (**C-4‴**) 32.43 (**C-1‴**) 21.19 ($\mathbf{CH_3}$–; overlapped) 16.74 (**C-6**; overlapped).

LRMS [M + Na]$^+$. Experimental: 501.2; calculated: 501.2.

*2.4.13. 1′,4′-Bis-C-(4-O-acetyl-2,3,6-trideoxy-α-L-erythro-heks-2-enpyranosyl)-but-2′-en (**4f**)*

E Isomer. ^{1}H NMR (600 MHz, $CDCl_3$) δ ppm: 5.92 (**H-2**; ddd; $J_{2\text{-}3} = 10.2$ Hz; $J_{2\text{-}1} = 2.2$ Hz; $J_{2\text{-}4} = 1.5$ Hz; 2H) 5.77 (**H-3**; ddd; $J_{3\text{-}2} = 10.2$ Hz; $J_{3\text{-}4} = 3.4$ Hz; $J_{3\text{-}1} = 2.2$ Hz; 2H) 5.58 (**H-2′**; ddddd; $J_{2'\text{-}3'} = 15.5$ Hz; $J_{2'\text{-}1'a} = 6.5$ Hz; $J_{2'\text{-}1'b} = 7.5$ Hz; $J_{2'\text{-}4'a} = J_{2'\text{-}4'b} = -1.5$ Hz; 1H) 5.58 (**H-3′**; ddddd; $J_{3'\text{-}2'} = 15.5$ Hz; $J_{3'\text{-}4'a} = 6.5$ Hz; $J_{3'\text{-}4'b} = 7.5$ Hz; $J_{3'\text{-}1'a} = J_{3'\text{-}1'b} = -1.5$ Hz; 1H) 4.88 (**H-4**; dddd; $J_{4\text{-}5} = 4.7$ Hz; $J_{4\text{-}3} = 3.4$ Hz; $J_{4\text{-}2} = 1.4$ Hz; $J_{4\text{-}1} = 2.2$ Hz; 2H) 4.17 (**H-1**; ddddd; $J_{1\text{-}1'a} = 9.0$ Hz; $J_{1\text{-}1'b} = 6.7$ Hz; $J_{1\text{-}2} = J_{1\text{-}3} = J_{1\text{-}4} = 2.2$ Hz; 2H) 3.91 (**H-5**; dq; $J_{5\text{-}6} = 6.6$ Hz × 3; $J_{5\text{-}4} = 4.7$ Hz; 2H) 2.46/2.40 (**H-1′a**, **H-4′a**; m; 2H) 2.28 (**H-1′b**; dddd; $J_{1'b\text{-}1'a} = 14.0$ Hz; $J_{1'b\text{-}2'} = 7.5$ Hz; $J_{1'b\text{-}1} = 6.7$ Hz; $J_{1'b\text{-}3'} = -1.5$ Hz; 1H) 2.28 (**H-4′b**; dddd; $J_{4'b\text{-}4'a} = 14.0$ Hz; $J_{4'b\text{-}3'} = 7.5$ Hz; $J_{4'b\text{-}1} = 6.7$ Hz; $J_{4'b\text{-}2'} = -1.5$ Hz; 1H) 2.09 ($\mathbf{CH_3}$; s; 6H) 1.23 (**H-6**; d; $J_{6\text{-}5} = 6.6$; 6H).

^{13}C NMR (150 MHz, $CDCl_3$) δ ppm: 16.92 (**C-6**) 21.18 ($\mathbf{CH_3}$) 37.17 (**C-1′**, **C-4′**) 68.62 (**C-4**) 69.57 (**C-5**) 70.13 (**C-1**) 122.94 (**C-3**) 128.66 (**C-2′**, **C-3′**) 133.46 (**C-2**) 170.69 (**C=O**).

Z Isomer. ^{1}H NMR (600 MHz, $CDCl_3$) δ ppm: 5.93 (**H-2**; ddd; $J_{2\text{-}3} = 10.2$ Hz; $J_{2\text{-}1} = 2.2$ Hz; $J_{2\text{-}4} = 1.4$ Hz; 2H) 5.79 (**H-3**; ddd; $J_{3\text{-}2} = 10.2$ Hz; $J_{3\text{-}4} = 3.5$ Hz; $J_{3\text{-}1} = 2.2$ Hz; 2H) 5.61 (**H-2′**; ddddd; $J_{2'\text{-}3'} = 11.0$ Hz; $J_{2'\text{-}1'a} = 6.5$; $J_{2'\text{-}1'b} = 7.5$ Hz; $J_{2'\text{-}4'a} = J_{2'\text{-}4'b} = -1.6$ Hz; 1H) 5.61 (**H-3′**; ddddd; $J_{3'\text{-}2'} = 11.0$ Hz; $J_{3'\text{-}4'a} = 6.5$ Hz; $J_{3'\text{-}4'b} = 7.5$ Hz; $J_{3'\text{-}1'a} = J_{3'\text{-}1'b} = -1.6$ Hz; 1H) 4.88 (**H-4**; dddd; $J_{4\text{-}5} = 4.9$ Hz; $J_{4\text{-}3} = 3.5$ Hz; $J_{4\text{-}2} = 1.4$ Hz; $J_{4\text{-}1} = 2.2$ Hz; 2H) 4.19 (**H-1**; ddddd; $J_{1\text{-}1'a} = 9.0$ Hz; $J_{1\text{-}1'b} = 6.7$ Hz; $J_{1\text{-}2} = J_{1\text{-}3} = J_{1\text{-}4} = 2.2$ Hz; 2H) 3.92 (**H-5**; dq; $J_{5\text{-}6} = 6.6$ Hz × 3; $J_{5\text{-}4} = 4.9$ Hz; 2H) 2.46/2.40 (**H-1′a**, **H-4′a**; overlapped; 2H) 2.34 (**H-1′b**; dddd; $J_{1'b\text{-}1'a} = 15.2$ Hz; $J_{1'b\text{-}2'} = 7.5$ Hz; $J_{1'b\text{-}1} = 6.7$ Hz; $J_{1'b\text{-}3'} = -1.6$ Hz; 1H) 2.34 (**H-4′b**; dddd; $J_{4'b\text{-}4'a} = 15.2$ Hz; $J_{4'b\text{-}3'} = 7.5$ Hz; $J_{4'b\text{-}1} = 6.7$ Hz; $J_{4'b\text{-}2'} = -1.6$ Hz; 1H) 2.09 ($\mathbf{CH_3}$; s; 6H) 1.23 (**H-6**; d; $J_{6\text{-}5} = 6.6$ Hz; 6H).

^{13}C NMR (150 MHz, $CDCl_3$) δ ppm: 16.92 (**C-6**) 21.18 ($\mathbf{CH_3}$) 32.11 (**C-1′**, **C-4′**) 68.74 (**C-4**) 69.52 (**C-5**) 69.92 (**C-1**) 123.06 (**C-3**) 127.45 (**C-2′**, **C-3′**) 133.39 (**C-2**) 170.69 (**C=O**).

HRMS [M + Na]$^+$. Experimental: 387.1760; calculated: 387.1778.

*2.4.14. 7′-O-(1‴-C-(4,6-Di-O-acetyl-2,3-dideoxy-α-D-erythro-heks-2-enpyranosyl)-but-2‴-en-4‴-yl)-genistein (**3g**)*

E Isomer. ^{1}H NMR (600 MHz, $CDCl_3$) δ ppm: 12.81 (**OH-5′**; s; 1H) 7.84 (**H-2′**; s; 1H) 7.36 (**H-2″**, **H-6″**; d; $J_{10'\text{-}11',14'\text{-}13'} = 8.63$ Hz; 2H) 6.87 (**H-3″**, **H-5″**; d; $J_{11'\text{-}10,13'\text{-}14'} = 7.96$ Hz; 2H)

6.39 (**H-8′**; d; $J_{5'\text{-}7'}$ = 2.65 Hz; 1H) 6.38 (**H-6′**; d; $J_{7'\text{-}5'}$ = 2.65 Hz; 1H) 5.96/5.79 (**H-2**, **H-3**, **H-2‴**, **H-3‴**; m; 4H) 5.14 (**H-4**; broad signal; 1H) 4.55 (**H-4‴**; d; $J_{4''\text{-}3''}$ = 5.97 Hz; 2H) 4.32 (**H-1**; ddd; $J_{1\text{-}1''a}$ = 7.8 Hz; $J_{1\text{-}1''b}$ = 5.47 Hz; $J_{1\text{-}2}$ = 2.65 Hz; 1H) 4.27 (**H-6a**; dd; $J_{6a\text{-}6b}$ = 11.94 Hz; $J_{6a\text{-}5}$ = 6.63 Hz; 1H) 4.16 (**H-6b**; dd; $J_{6b\text{-}6a}$ = 11.61 Hz; $J_{6b\text{-}5}$ = 3.65 Hz; 1H) 3.98 (**H-5**; ddd; $J_{5\text{-}6a}$ = 6.3 Hz; $J_{5\text{-}4}$ = 6.3 Hz; $J_{5\text{-}6b}$ = 3.98 Hz; 1H) 2.52 (**H-1‴a**; ddd; $J_{1''a\text{-}1''b}$ = 13.93 Hz; $J_{1''a\text{-}1}$ = 6.97 Hz; $J_{1''a\text{-}2''}$ = 6.97 Hz; 1H) 2.39 (**H-1‴b**; ddd; $J_{1''a\text{-}1''b}$ = 14.60 Hz; $J_{1''b\text{-}1}$ = 5.97 Hz; $J_{1''b\text{-}2''}$ = 5.97 Hz; 1H) 2.10 (**OAc**; s; 6H).

^{13}C NMR (150 MHz, $CDCl_3$) δ ppm: 180.87 (**C-4′**) 170.96 (**C=O**) 170.53 (**C=O**) 164.44 (**C-7′**) 162.57 (**C-4′**) 157.88 (**C-4″**) 156.18 (**C-8′a**) 152.76 (**C-2′**) 132.59 (**C-2**) 131.07 (**C-2‴**) 130.23 (**C-2″**, **C-6″**) 126.83 (**C-3‴**) 123.90 (**C-3**) 123.68 (**C-3′**) 122.60 (**C-1″**) 115.61 (**C-3″**, **C-5″**) 106.23 (**C-4′a**) 98.74 (**C-6′**) 93.08 (**C-8′**) 71.16 (**C-1**) 69.91 (**C-5**) 68.87 (**C-4‴**) 64.87 (**C-4**) 62.77 (**C-6**) 36.60 (**C-1‴**) 21.08 (**CH$_3$**–) 20.83 (**CH$_3$**–).

Z Isomer. ^{1}H NMR (600 MHz, $CDCl_3$) δ ppm: 12.82 (**OH-5′**; s; 1H) 7.85 (**H-2′**; s; 1H) 7.36 (**H-2″**, **H-6″**) 6.87 (**H-3″**, **H-5″**; 2H; overlapped) 6.41 (**H-8′**; d; $J_{5'\text{-}7'}$ = 2.65 Hz; 1H) 6.37 (**H-6′**; d; $J_{7'\text{-}5'}$ = 2.65 Hz; 1H) 5.96–5.79 (**H-2**, **H-3**, **H-2‴**, **H-3‴**; m; 4H; overlapped) 5.14 (**H-4**; broad signal; 1H; overlapped) 4.66 (**H-4‴**; d; $J_{4''\text{-}3''}$ = 5.31 Hz; 2H) 4.32 (**H-1**; 1H; overlapped) 4.29 (**H-6a**; d; $J_{6a\text{-}6b}$ = 11.94 Hz; 1H; partially overlapped) 4.16 (**H-6b**; 1H; overlapped) 4.01 (**H-5**; ddd; $J_{5\text{-}6a}$ = 6.47 Hz; $J_{5\text{-}4}$ = 6.47 Hz; $J_{5\text{-}6b}$ = 3.65 Hz; 1H) 2.52 (**H-1‴a**; 1H; overlapped) 2.46 (**H-1‴b**; ddd; $J_{1''b\text{-}1''a}$ = 14.60 Hz; $J_{1''b\text{-}1}$ = 5.97 Hz; $J_{1''b\text{-}2''}$ = 5.97 Hz; 1H) 2.10 (**OAc**; s; 6H).

^{13}C NMR (150 MHz, $CDCl_3$) δ ppm: 180.87 (**C-4′**; overlapped) 170.96 (**C=O**; overlapped) 170.53 (**C=O**; overlapped) 164.44 (**C-7′**; overlapped) 162.57 (**C-5′**; overlapped) 157.88 (**C-4″**; overlapped) 156.18 (**C-8′a**; overlapped) 152.76 (**C-2′**; overlapped) 132.50 (**C-2**) 130.23 (**C-2″**, **C-6″**; overlapped) 129.88 (**C-2‴**) 126.30 (**C-3‴**) 124.02 (**C-3**) 123.68 (**C-3′**; overlapped) 122.60 (**C-4′a**; overlapped) 115.61 (**C-3″**, **C-5″**; overlapped) 106.23 (**C-3′**; overlapped) 98.68 (**C-6′**) 93.11 (**C-8′**) 70.93 (**C-1**) 70.15 (**C-5**) 64.55 (**C-4‴**) 64.87 (**C-4**; overlapped) 62.77 (**C-6**; overlapped) 32.11 (**C-1‴**) 21.08 (**CH$_3$**; overlapped) 20.83 (**CH$_3$**; overlapped).

HRMS [M + Na]$^+$. Experimental: 559.1560; calculated: 559.1575.

2.4.15. *1′,4′-Bis-C-(4,6-di-O-acetyl-2,3-dideoxy-α-D-erythro-heks-2-enpyranosyl)-but-2′-en (**4g**)*

E Isomer. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 5.93 (**H-2**; ddd; $J_{2\text{-}3}$ = 10.45 Hz; $J_{2\text{-}1}$ = 2.41 Hz; $J_{2\text{-}4}$ = 1.51 Hz; 2H) 5.80 (**H-3**; ddd; $J_{3\text{-}2}$ = 10.30 Hz; $J_{3\text{-}4}$ = 2.83 Hz; $J_{2\text{-}1}$ = 2.08 Hz; 2H) 5.58 (**H-2′**, **H-3′**; t; 3.7 Hz × 2; 2H) 5.14 (**H-4**; m; 2H) 4.26 (**H-6a**; dd; $J_{6a\text{-}6b}$ = 11.92 Hz; $J_{6a\text{-}5}$ = 6.32 Hz; 2H) 4.26 (**H-1**; overlapped przez **H-6a**; 2H) 4.14 (**H-6b**; ddd; $J_{6b\text{-}6a}$ = 12.06 Hz; $J_{6b\text{-}5}$ = 3.59 Hz; 2H) 3.95 (**H-5**; ddd; $J_{5\text{-}6a}$ = 6.43 Hz; $J_{5\text{-}4}$ = 6.43; $J_{5\text{-}6b}$ = 3.38 Hz; 2H) 2.49/2.25 (**H-1′a**, **H-1′b**, **H-4′a**, **H-4′b**; m; 4H) 2.10 (–**CH$_3$**; s; 12H).

^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 170.83 (**C=O**) 170.42 (**C=O**) 132.85 (**C-2**) 128.61 (**C-2′**, **C-3′**) 123.65 (**C-3**) 71.64 (**C-5**) 69.83 (**C-1**) 64.92 (**C-4**) 62.79 (**C-6**) 36.77 (**C-1′**, **C-4′**) 21.10 (**CH$_3$**) 20.85 (**CH$_3$**).

Z Isomer. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 5.94 (**H-2**; ddd; $J_{2\text{-}3}$ = 10.45 Hz; $J_{2\text{-}1}$ = 2.41 Hz; $J_{2\text{-}4}$ = 1.36 Hz; 2H) 5.82 (**H-3**; ddd; $J_{3\text{-}2}$ = 10.30 Hz; $J_{3\text{-}4}$ = 2.84 Hz; $J_{2\text{-}1}$ = 2.08 Hz; 2H) 5.61 (**H-2′**, **H-3′**; t; 4.9 Hz × 2; 2H) 5.14 (**H-4**; m; 2H) 4.26 (**H-6a**; overlapped; 2H) 4.26 (**H-1**; overlapped; 2H) 4.14 (**H-6b**; overlapped; 2H) 3.96 (**H-5**; ddd; $J_{5\text{-}6a}$ = 6.57 Hz; $J_{5\text{-}4}$ = 6.57 Hz; $J_{5\text{-}6b}$ = 3.23 Hz; 2H) 2.49–2.25 (**H-1′a**, **H-1′b**, **H-4′a**, **H-4′b**; m; 4H) 2.09 (–**CH$_3$**; s; 12H).

^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 170.83 (**C=O**; overlapped) 170.42 (**C=O**; overlapped) 132.77 (**C-2**) 127.30 (**C-2′**, **C-3′**) 123.77 (**C-3**) 71.39 (**C-5**) 69.98 (**C-1**) 64.92 (**C-4**; overlapped) 62.79 (**C-6**; overlapped) 31.71 (**C-1′**, **C-4′**) 21.10-20.85 (**CH$_3$**; overlapped).

HRMS [M + Na]$^+$. Experimental: 503.1906; calculated: 503.1888.

2.4.16. *7′-O-(1‴-C-(4,6-Di-O-acetyl-2,3-dideoxy-α-D-treo-heks-2-enpyranosyl)-but-2‴-en-4‴-yl)-genistein (**3h**)*

Isomer E. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 12.81 (**H-5′**; s; 1H) 7.83 (**H-2′**; s; 1H) 7.35/7.33 (**H-2″**, **H-6″**; m; 2H) 6.87/6.84 (**H-3″**; **H-5″**; m; 2H) 6.39 (**H-8′**; d; $J_{5'\text{-}7'}$ = 2.3 Hz; 1H) 6.35 (**H-6′**; d; $J_{7'\text{-}5'}$ = 2.3 Hz; 1H) 6.07–5.98 (**H-2**, **H-3**; m; 2H) 5.91 (**H-2‴**; ddd; $J_{2''\text{-}3''}$ = 15.6 Hz; $J_{2''\text{-}1''a}$ = 6.5 Hz; $J_{2''\text{-}1''b}$ = 6.4 Hz; 1H) 5.80 (**H-3‴**; dt; $J_{3''\text{-}2''}$ = 15.6 Hz; $J_{3''\text{-}4''}$ = 5.3 Hz × 2; 1H) 5.09 (**H-4**; dd; $J_{4\text{-}3}$ = 4.3 Hz; $J_{4\text{-}5}$ = 2.7 Hz; 1H) 4.54 (**H-4‴**; d; $J_{4''\text{-}3''}$ = 5.3 Hz; 1H) 4.42–4.37 (**H-1**; m; 1H) 4.24/4.21 (**H-6a**, Hz; $J_{1''a\text{-}1}$ = 8.3 Hz; 1H) 2.35 (**H-1‴b**; ddd; $J_{1''b\text{-}1''a}$ = 14.6 Hz; $J_{1''b\text{-}2''}$ = 6.4 Hz; $J_{1''b\text{-}1}$ = 5.7 Hz; 1H) 2.09 (–**CH$_3$**; s; 3H) 2.09 (–**CH$_3$**; s; 3H).

^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 20.79 (–**CH$_3$**) 20.87 (–**CH$_3$**) 35.22 (**C-1‴**) 62.89 (**C-6**) 63.80 (**C-4**) 68.07 (**C-5**) 68.80 (**C-4‴**) 72.11 (**C-1**) 93.03 (**C-8′**) 98.74 (**C-6′**) 106.19 (**C-4′a**) 115.62 (**C-3″**, **C-5″**) 122.27 (**C-3**) 122.49 (**C-1″**) 123.67 (**C-3′**) 126.94 (**C-3‴**) 130.18 (**C-2″**, **C-6″**) 130.84 (**C-2‴**) 134.37 (**C-2**) 152.79 (**C-2′**) 156.34 (**C-5′**) 157.82 (**C-8′a**) 162.47 (**C-4″**) 164.42 (**C-7′**) 170.70 (**C=O**) 170.98 (**C=O**) 180.88 (**C-4′**).

Z Isomer. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 12.81 (**H-5′**; s; 1H) 7.85 (**H-2′**; s; 1H) 7.35/7.33 (**H-2″**, **H-6″**; overlapped; 2H) 6.87/6.84 (**H-3″**; **H-5″**; overlapped; 2H) 6.40 (**H-8′**; d; $J_{5'\text{-}7'}$ = 2.3 Hz; 1H) 6.36 (**H-6′**; d; $J_{7'\text{-}5'}$ = 2.3 Hz; 1H) 6.07/5.98 (**H-2**, **H-3**; overlapped; 2H) 5.85/5.76 (**H-2‴**, **H-3‴**; overlapped; 1H) 5.12 (**H-4**; dd; $J_{4\text{-}3}$ = 4.0 Hz; $J_{4\text{-}5}$ = 2.2 Hz; 1H) 4.65 (**H-4‴**; d; $J_{4''\text{-}3''}$ = 4.6 Hz; 1H) 4.42/4.37 (**H-1**; overlapped; 1H) 4.24/4.21 (**H-6a, H-6b**; overlapped; 2H) 4.18/4.13 (**H-5**; overlapped; 1H) 2.57/2.43 (**H-1‴a**, **H-1‴b**; overlapped; 2H) 2.10 (–**CH$_3$**; s; 3H) 2.08 (–**CH$_3$**; s; 3H).

^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 20.79 (–**CH$_3$**, overlapped) 20.87 (–**CH$_3$**, overlapped) 31.04 (**C-1‴**) 62.81 (**C-6**) 63.80 (**C-4**) 64.43 (**C-4‴**) 68.18 (**C-5**, overlapped) 71.92 (**C-1**) 93.03 (**C-8′**, overlapped) 98.68 (**C-6′**) 106.24 (**C-4′a**) 115.62 (**C-3″**, **C-5″**, overlapped) 122.39 (**C-3**) 122.49 (**C-1″**, overlapped) 123.67 (**C-3′**, overlapped) 126.24 (**C-3‴**) 129.87

(**C-2‴**) 130.18 (**C-2″**, **C-6″**, overlapped) 134.16 (**C-2**) 152.79 (**C-2′**, overlapped) 156.34 (**C-5′**, overlapped) 157.82 (**C-8′a**, overlapped) 162.517 (**C-4″**) 164.39 (**C-6′**) 170.70 (**C=O**, overlapped) 170.98 (**C=O**, overlapped) 180.88 (**C-4′**, overlapped).

HRMS [M + Na]$^+$. Experimental: 559.1552; calculated: 559.1575.

*2.4.17. 1′,4′-Bis-C-(4,6-di-O-acetyl-2,3-dideoxy-α-D-treo-heks-2-enpyranosyl)-but-2′-en (**4h**)*

E Isomer. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 6.04 (**H-2** dd; $J_{2\text{-}3}$ = 10.2 Hz; $J_{2\text{-}1}$ = 2.7 Hz; 2H) 5.98 (**H-3**; ddd; $J_{3\text{-}2}$ = 10.2 Hz; $J_{3\text{-}4}$ = 4.7 Hz; $J_{3\text{-}1}$ = 1.7 Hz; 2H) 5.59 (**H-2′**, **H-3′**; t; 3.8 Hz × 2; 2H) 5.08 (**H-4**; dd; $J_{4\text{-}3}$ = 4.7 Hz; $J_{4\text{-}5}$ = 2.3 Hz; 2H) 4.32 (**H-1**; dddd; $J_{1\text{-}1'a}$ = 7.8; $J_{1\text{-}1'b}$ = 5.2 Hz; $J_{1\text{-}2}$ = 2.7 Hz; $J_{1\text{-}3}$ = 1.7 Hz; 2H) 4.21/4.19 (**H-6a, H-6b**; m; 4H) 4.16–4.10 (**H-5**; m; 2H) 2.46–2.35 (**H-1′a**, **H-4′a**; m; 2H) 2.31/2.23 (**H-1′b**, **H-4′b**; m; 2H) 2.09 (–**CH**$_3$; s; 6H) 2.08 (–**CH**$_3$; s; 6H).

^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 20.81 (–**CH**$_3$) 20.90 (–**CH**$_3$) 35.72 (**C-1′**, **C-4′**) 62.86 (**C-6**) 63.77 (**C-4**) 68.04 (**C-5**) 72.58 (**C-1**) 122.04 (**C-3**) 128.53 (**C-2′**, **C-3′**) 134.69 (**C-2**) 170.52 (**C=O**) 170.70 (**C=O**).

Z Isomer. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 6.06 (**H-2**; dd; $J_{2\text{-}3}$ = 10.4 Hz; $J_{2\text{-}1}$ = 2.9 Hz; 2H) 5.98 (**H-3**; overlapped; 2H) 5.63 (**H-2′**; t; 4.8 Hz × 2; 2H) 5.08 (**H-4**; overlapped; 2H) 4.32 (**H-1**; overlapped; 2H) 4.21/4.19 (**H-6a**, **H-6b**; overlapped; 4H) 4.16/4.10 (**H-5**; overlapped; 2H) 2.46/2.35 (**H-1′a**, **H-1′b**, **H-4′a**, **H-4′b**; overlapped; 4H) 2.09/2.08 (–**CH**$_3$; overlapped; 12H).

^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 20.81 (–**CH**$_3$, overlapped) 20.90 (–**CH**$_3$, overlapped) 30.66 (**C-1′**, **C-4′**) 62.86 (**C-6**, overlapped) 63.74 (**C-4**) 68.09 (**C-5**) 72.32 (**C-1**) 122.16 (**C-3**) 127.23 (**C-2′**, **C-3′**) 134.59 (**C-2**) 170.52 (**C=O**, overlapped) 170.70 (**C=O**, overlapped).

HRMS [M + Na]$^+$. Experimental: 503.1911; calculated: 503.1888.

2.5. Cancer Cell Lines. HCT 116 colorectal cancer and DU 145 prostate cancer cell lines obtained from ATCC (American Type Culture Collection, Rockville, MD, USA) were cultured in RPMI 1640 medium supplemented with 10% of fetal bovine serum (FBS) (MP Biomedicals) and 1 μg/mL gentamicin (KRKA, Novo Mesto, Slovenia), at 37°C in a humidified atmosphere containing 5% CO_2 in the air. Cells were split at 90% confluence. The grown cells were detached by rinsing with 0.02% ethylenediamine tetraacetic acid (EDTA) followed by 0.25% trypsin.

2.6. Cytotoxicity Assays. Cell viability was estimated using an MTT (3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazolium bromide) assay (Sigma-Aldrich, Germany), according to the supplier's protocol. After 72 h treatment with tested agents (**3a** and **3f**) at concentration series: 0.1, 1, 5, 10, 25, and 50 μM the medium was aspirated, and cells were incubated for 3 hours at 37°C with 0.5 mg/mL MTT solution (50 μL) in Dulbecco modified essential medium (DMEM) (Sigma-Aldrich, Germany) without phenol red. Then the medium was aspired, insoluble crystals of formazan were solubilized in 2-propanol: HCl solution, and optical density (λ = 570 nm) was determined in a microplate reader BioTek Synergy II (BioTek Instruments, USA). IC_{50} was estimated using CalcuSyn software using the 4 parameter nonlinear regression model for curve-fitting analysis. The experiments were repeated at least three times.

2.7. Cell Cycle Analysis and Microscopy Studies. Cell cycle analysis was performed after 24 h treatment of cells with the tested compounds with FACSCanto flow cytometer as described elsewhere [17]. Microscope analysis of cell morphology was performed with use of inverted OLYMPUS IX 70 microscope (Olympus, Japan) equipped with differential interference contrast. Confocal images of cells treated with the **3a** and **3f** derivatives were taken with Zeiss LSM 710 (Carl Zeiss, Germany) with use of 63x oil objective. For nuclei visualization cells were treated with 5 μg/mL Hoechst 33342 (Sigma-Aldrich, Germany) for 90 minutes, and for lysosomes and mitochondria staining cells were treated with 100 nM Lysotracker red and 200 nM Mitotracker green (both from Molecular Probes, USA) for 30 minutes.

2.8. Determination of Compounds Stability. 50% HCO_2H in H_2O for HPLC were supplied by Sigma-Aldrich, Germany, and MeCN and H_2O for LC-MS were supplied by POCH, Poland. For analytes preparation HCT 116 cells were plated in 24-well plate (at 25% confluence). Cells were cultured at 37°C in humidified atmosphere of 5% CO_2 for 48 h. The culture media consisted of RPMI, 10% fetal bovine serum, and 0.1% gentamycin sulfate. After 48 h the culture medium was removed from wells and replaced by 1 mL 10 μM solution of **3a** and **3f** in a fresh medium. Cells were incubated for additional 8 h or 24 h. Then the medium was collected and centrifuged (2000 g, 2 min). The supernatant was filtrated through a syringe filter (0.22 μL, RC) and transferred to an autosampler vial. The supernatant was analyzed by LC-MS/MS. In control experiments, compounds were incubated with medium without cells (RPMI supplemented with 10% fetal bovine serum and 0.1% gentamycin sulfate). The LC separation was performed using a Dionex UHPLC system (Dionex Corporation, Sunnyvalem, CA, USA) consisting of an UltiMate 3000 RS pump, an UltiMate 3000 autosampler, an UltiMate 3000 column compartment, and an UltiMate 3000 variable wavelength detector. UHPLC system was operated using Dionex Chromaleon 6.8 software. Chromatography was performed using a C18 ACE column (150 × 4.6 mm, 3.0 μm, Advance Chromatogr. Technologies, Aberdeen, UK) connected by integral holder (3.2 × 4.6 mm) with guard column of the same material. Isocratic conditions were applied: 25% of 0.1% aq. HCO_2H and 75% of MeCN, the flow rate was set at 0.3 mL/min, and sample injection volume was 5 μL. The HPLC column was thermostated at 25°C, and samples were kept in the autosampler at 10°C. Compounds were detected at λ 260 nm. In order to determine the mass of compounds, the UHPLC system was connected to a 4000 Q TRAP triple quadrupole linear ion trap mass spectrometer

Table 1: CM reactions of C-allyl glycosides (**1a**–**1h**) and exo-glycal (**1i**) with 7-O-allylgenistein (**2**).

Entry	Sugar	Glycoconjugate Yields[a] (E/Z)[c]	C-Allyl glycoside homodimer Yields (%)[a] (E/Z)[c]	7-O-Allylgenistein homodimer Yields (%)[b] (E/Z)[c]
1	**1a**	**3a** 42% (2.5) 25% (10)[d]	**4a** 7% (2.5) 18% (only E)[d]	**5** 57% (2.0) 47% (11.4)[d]
2	**1b**	**3b** 41% (2.1)	**4b** 29% (1.8)	**5** 36% (2.0)
3	**1c**	**3c** 44% (2.2)	**4c** 30% (n.d.)	**5** 43% (2.0)
4	**1d**	**3d** 18% (2.6)	**4d** 6% (2.0)	**5** 30% (2.0)
5	**1e**	**3e** 33% (2.1)	**4e** 9% (1.5)	**5** 47% (2.0)
6	**1f**	**3f** 20% (2.2)	**4f** 8% (1.6)	**5** 13% (2.0)
7	**1g**	**3g** 4% (2.4)	**4g** 13% (1.5)	**5** 28% (2.0)
8	**1h**	**3h** 35% (2.8) 17% (7.3)[d]	**4h** 26% (2.4) 5% (only E)[d]	**5** 29% (2.0) 18% (11.4)[d]
9	**1i**	—	—	**5** 21% (2.0)

[a]Yields calculated according to initial amount of C-allyl glycoside.
[b]Yields calculated according to initial amount of allylgenistein.
[c]E/Z molar ratio was calculated from ^{1}H NMR spectra.
[d]CM reactions initiated by II generation Grubbs complex.

(Applied Biosystem/MDS SCIEX, Foster City, CA, USA). For data acquisition, Analyst software (version 1.4) was used. The optimum mass spectrometer parameters for the detection of compounds were as follows: ion spray voltage (IS) at 4000 V, ion source gas 1 (GS1) at 40 psi, ion source gas 2 (GS2) at 60 psi, curtain gas (CUR) at 10 psi, declustering potential (DP) at 90 V, and entrance potential (EP) 10 V. The source temperature was set for 600°C. As the ion source gas and collision gas high-pressure N_2 was used. The mass spectrometer operated in the positive electrospray ionization (ESI) mode. Full scan mass spectra were acquired from *m/z* 1 to 700 in 1 s cycle time.

3. Results and Discussion

Terminal olefins were synthetized by well-known procedures: C-allyl glycosides (**1a**–**1 h)** (Figure 1) were obtained in reactions with trimethylallylsilane catalyzed by Lewis acid [18–20], and 1-C-methylidene-D-glucopyranoside (**1i**) was obtained via Ramberg-Backlund rearrangement [18, 19], 7-O-allylgenistein (**2**) was synthesized from tetrabutyl ammonium salt of genistein and allyl bromide [20].

3.1. Cross-Metathesis Reactions. The presented reactions (Figure 1) were promoted by first and second generation Grubbs catalysts (Table 1). When exposed to air or complexing agents, Grubbs' complexes deactivate or change their catalytic behavior [21]. Thus Schlenck type apparatus and high purity of reactants were used to conduct reactions with high yield. In our experiments we utilized suspensions of Grubbs catalysts in paraffin, since handling such prepared initiator is much easier [22]; it can be stored without inert gas protection and weighted with higher precision. All reactions were performed under Ar atmosphere in refluxing CH_2Cl_2. A closely related problem is the removal of the Ru complex and products of its decomposition from the reaction mixture [23, 24]. The formed genistein glycoconjugates have had to be chromatographed at least twice in order to get colorless substances.

The result of a CM reaction can be described by several parameters: overall yield, which depends on initiator stability, selectivity, which can be described by the ratio of homodimers to a CM product ($1 : n_{GG}/n_{FG} : n_{FF}/n_{FG}$), and stereoselectivity, which can be described by the molar ratio of E/Z isomers.

The observed turnover numbers were between 4 and 22, whereas, according to the known literature, usual turnover numbers are between 5 and 20 [25–30]. If turnover number is insufficient to provide complete conversion of a substrate, a common method is to increase the amount of metathesis initiator. However, this approach has a few disadvantages, such as the increase of the cost of the synthesis and the increase of the amount of impurities originating from Ru complex decomposition. Therefore, we decided to use no more than 6 mol % of initiator according to the amount of C-allyl glycoside.

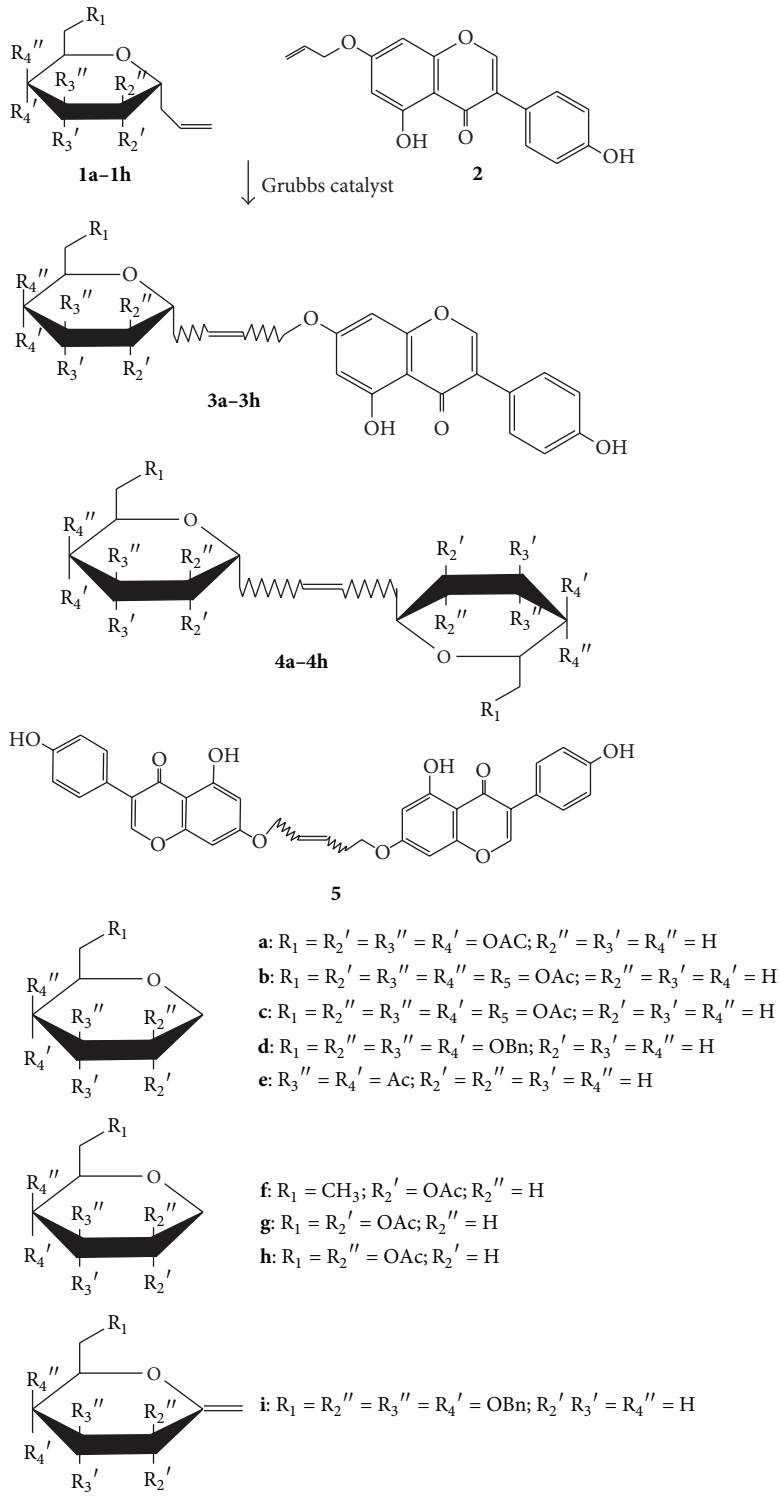


Figure 1: Olefin cross-metathesis reaction between C-allylglycoside (**1a–1h**) and 7-O-allylgenistein (**2**).

Another problem of CM reactions is their selectivity. In reactions described herein, six products were obtained from only two substrates (Figure 1). The selectivity of the process is decreased by the formation of homodimers (compounds **4** and **5**) and can be predicted if the ability of olefins to undergo homodimerisation is known. Grubbs and coworkers divided terminal olefins into four groups, according to their reactivity in CM [31]: type I olefins undergo a rapid homodimerization, and their homodimers are consumable in the reaction; type II olefins undergo a slow homodimerization

and their homodimers are also consumable in a much lower rate; type III olefins do not undergo homodimerisation and participate in the reaction only if an olefin of type I or II is present; and type IV olefins do not participate in the reaction at all. When two olefins of the same type are reacted with each other, formation of all possible metathesis products can be observed. However, when an olefin of high reactivity (type I) is reacted with excess of an olefin which undergoes a slow or no homodimerization (type II or III), selective CM can be achieved. Therefore we made attempts to establish the reactivity of the utilized C-allyl glycosides and 7-O-allylgenistein. In CM reactions initiated by first generation Grubbs catalysts olefins **1a** to **1h** are type II olefins. They do undergo homodimerisation; however, their homodimers do not participate in CM (Table 1, entries: 1–8). Compound **1i** is type IV olefin. When reacted with **2**, only formation of homodimer **5** could be observed. Grubbs and coworkers have reported unusually high reactivity of α, β-unsaturated carbonyl compounds like acrylic acid [32]. Therefore, we have conducted an experiment with the second generation Ru catalyst, **1i** and acrylic acid. Unfortunately not even traces of a CM product could be found in the reaction mixture. Most of the presented experiments are CM reactions between two olefins of second type. Such reactions are examples of nonselective CM reactions, and all possible products can be formed.

In contrast to ring closing metathesis (RCM) reactions [33, 34], CM reactions usually suffer from poor stereoselectivity. Products of olefin cross-metathesis reactions were obtained as mixtures of E/Z isomers. The ratio of E/Z isomers was about 2 when first generation of Grubbs catalysts was used. Some improvement was achieved with second generation of Grubbs catalysts used as a metathesis initiator. The ratio of E/Z isomers increased then to 10 and 7.3, but the yields were significantly lower (Table 1, entries 1 and 8, resp.). Whether the observed improvement was due to secondary CM [35, 36] is a matter of question. In our experiments we did not reach complete conversion of substrates, thus CM reactions of products should be inhibited by the presence of the more reactive substrates. On the other hand Grubbs and coworkers have shown that at low conversions, reactions initiated by second generation catalysts gave a product with low E/Z ratio [35, 36].

3.2. Preliminary Results on Biological Activity and Stability in Culture Medium. For evaluation of antiproliferative activity of the new C-glycosidic derivatives of genistein two compounds, **3a** and **3f**, the analogs of previously tested glycoconjugates Glu-3 and Ram-3, respectively, [17] were selected. We performed a standard MTT assay in two human cancer cell lines: prostate cancer-derived DU 145 and colon cancer-derived HCT 116.

The derivatives showed the ability to inhibit the proliferation of cells in a dose-dependent manner in both cell lines, although DU 145 was less sensitive than HCT 116. The activity of the genistein derivatives, expressed as IC_{50} (the concentration necessary to inhibit the proliferation of cells to 50% of control) (Table 2), was higher than activity of genistein [17]. The results clearly show that the C-glycosides of genistein described herein have enhanced antiproliferative activity in comparison to a parent compound, like their O-glycosidic counterparts.

Table 2: IC_{50} values of compounds **3a** and **3f** measured by MTT assay after 72 h treatment of DU 145 and HCT 116 cell lines.

	IC_{50} value (μM)	
	DU 145	HCT 116
3a	9.50 ± 2.21	4.20 ± 1.11
3f	24.22 ± 6.77	8.99 ± 2.41
Genistein*	47.29 ± 11.78	34.90 ± 9.84

*Data from [17].

Table 3: Retention times and masses analyzed for the genistein C-glycosides.

Analyte	Retention time (min)	m/z
	Standards	
3a	7.93; 8.25	655
3f	10.7; 11.01	479
	Compound incubated in cell culture medium	
3a	7.97; 8.28	655
3f	7.14; 7.49	437

Microscope observations showed that cells treated with **3a** and **3f** were rounded and vacuolated (Figures 2(a), 2(c), and 2(e)). They detached easily from the bottom of a dish and floated in the culture medium. The confocal analysis showed that mitochondria and lysosomes were apparently unaffected, while the structure of nuclei in many cells was altered. Significant number of cells had fragmented nuclei (Figures 2(b), 2(d), and 2(f)). This observation suggested that the tested compounds acted in a similar manner to other genistein glycoconjugates which disturbed the mitotic apparatus [17].

In the next step we evaluated the influence of C-glycoconjugates on the cell cycle. The C-glycosidic derivatives described herein inhibited profoundly the cell cycle at G2/M phase in both cell lines tested: HCT 116 and DU 145 (Figure 3). The mode of action of the tested compounds resembled the mode of other previously described genistein derivatives substituted with modified rhamnal *via* a three-carbon atom linker containing O-glycosidic bond [17].

3.3. Stability of Compounds in Culture Media. In the next step we performed an analysis of stability of the tested compounds in culture media with and without cells after different duration of incubation. Growth media were collected from the culture dishes containing cultures of HCT 116 cells after 8 or 24 h treatment with 10 μM **3a** and **3f**. In control samples the tested compounds were incubated only with cell culture medium RPMI, containing 10% fetal bovine serum and 0.1% gentamycin sulfate. The analytes were analyzed by LC-MS/MS.

Based on analyses of mass spectra (Table 3) we found that the structure of **3a** is not altered after the incubation in

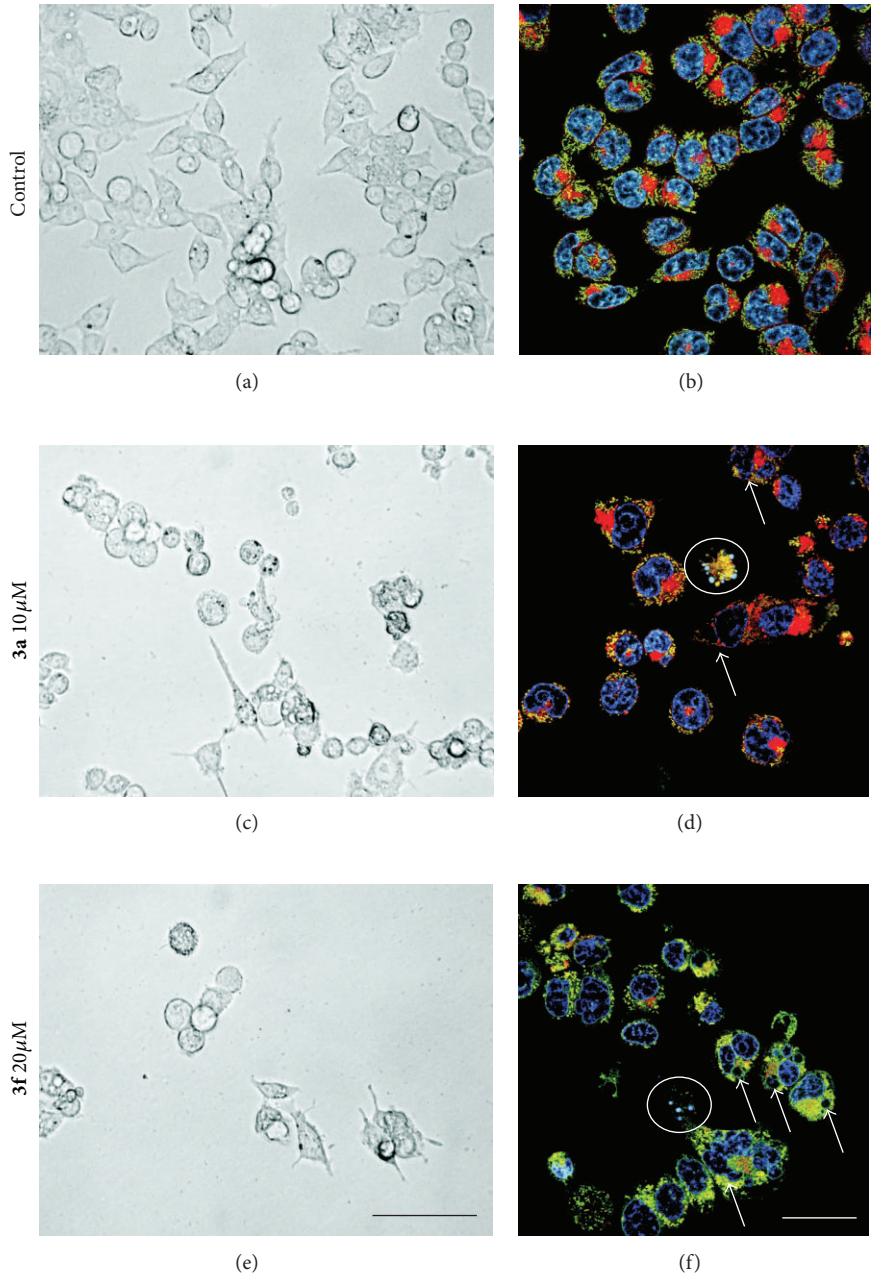


Figure 2: Morphology of HCT 116 cells treated with C-glycoconjugates of genistein for 24 h. (a), (b) Non-treated control; (c), (d) 5 μM **3a**; (e), (f) 20 μM **3f**. ((a), (c), and (e) DIC contrast; (b), (d), and (f) confocal images of cells stained with Hoechst 33342, Lysotracker red, and Mitotracker green). Circles in (d) and (f) mark apoptotic cells, arrows indicate cell with fragmented nuclei or with large micronuclei. Scale bar: 40 μm in left panel ((a), (c), and (e)); 20 μm in the right panel ((b), (d), and (f)).

cell culture medium (in both cell-free medium and medium collected from the cell cultures). In contrast, **3f** was less stable in culture medium (both in control medium and the medium aspirated from cell cultures). On the basis of the difference of the mass of the compound before and after incubation in culture media, we propose that the protecting acetyl group had detached. Besides of this minor structural change, the basic skeleton of a molecule was not altered during the incubation of this compound in culture medium.

4. Conclusions

It has been shown that application of olefin CM reaction initiated by first and second generation of Grubbs catalysts is an useful approach for the synthesis of new genistein glycoconjugates. *In vitro* experiments in cancer cells have shown that C-glycosidic derivatives of genistein exhibited similar mechanism of action to their O-glycosidic counterparts. These compounds were stable in a culture medium.

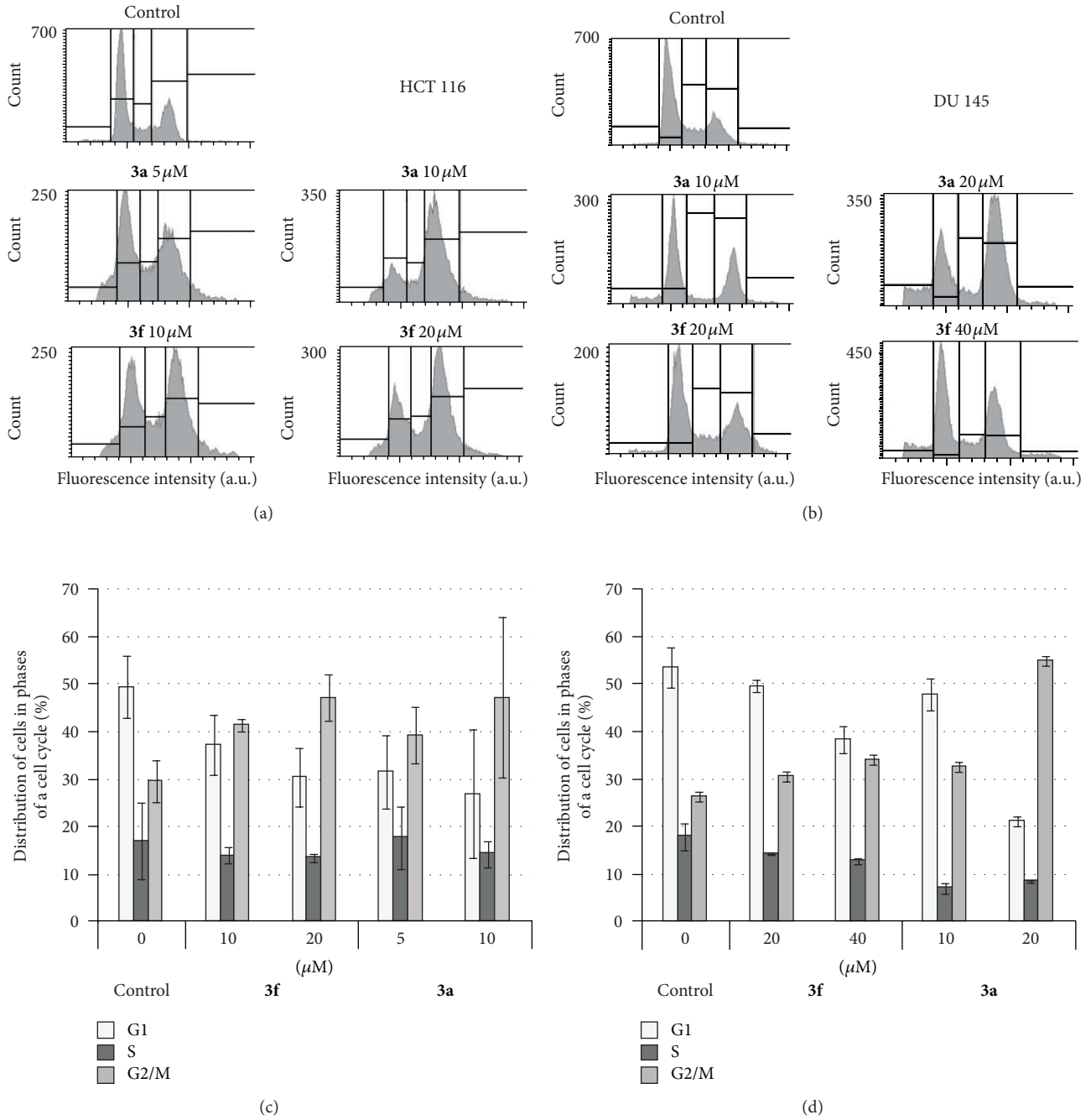


Figure 3: Cell cycle phases distribution in HCT 116 cells ((a), (c)) and DU 145 ((b), (d)) treated for 24 h with **3a** and **3f** used at the concentrations corresponding to their cytotoxic IC_{50} and $2xIC_{50}$. (a), (b) Representative histograms: (c), (d) mean values and standard deviations of percentage of cells in different phases of a cell cycle. a.u.; arbitrary units.

Acknowledgments

Research studies were part-financed by the European Union within the European Regional Development Fund (POIG.01.01.02-14-102/09, POIG.02.01.00-00-166/08). Ph.D. scholarship of KP was provided by the European Union Structural Funds in Poland (UDA-POKL.04.01.01-00-114/09-01).

References

[1] K. Szkudelska and L. Nogowski, "Genistein-A dietary compound inducing hormonal and metabolic changes," *Journal of Steroid Biochemistry and Molecular Biology*, vol. 105, no. 1–5, pp. 37–45, 2007.

[2] S. Banerjee, Y. Li, Z. Wang, and F. H. Sarkar, "Multi-targeted therapy of cancer by genistein," *Cancer Letters*, vol. 269, no. 2, pp. 226–242, 2008.

[3] V. B. Gencel, M. M. Benjamin, S. N. Bahou, and R. A. Khalil, "Vascular effects of phytoestrogens and alternative menopausal hormone therapy in cardiovascular disease," *Mini Reviews in Medicinal Chemistry*, vol. 12, no. 2, pp. 149–174, 2012.

[4] J. Lappe, I. Kunz, I. Bendik et al., "Effect of a combination of genistein, polyunsaturated fatty acids and vitamins D3 and K1 on bone mineral density in postmenopausal women: a randomized, placebo-controlled, double-blind pilot study," *European Journal of Nutrition*, vol. 52, no. 1, pp. 203–215, 2013.

[5] A. Cassidy, "Potential risks and benefits of phytoestrogen-rich diets," *International Journal for Vitamin and Nutrition Research*, vol. 73, no. 2, pp. 120–126, 2003.

[6] H. Marini, L. Minutoli, F. Polito et al., "Effects of the phytoestrogen genistein on bone metabolism in osteopenic postmenopausal women: a randomized trial," *Annals of Internal Medicine*, vol. 146, no. 12, pp. 839–847, 2007.

[7] A. Rusin, Z. Krawczyk, G. Grynkiewicz, A. Gogler, J. Zawisza-Puchałka, and W. Szeja, "Synthetic derivatives of genistein, their properties and possible applications," *Acta Biochimica Polonica*, vol. 57, no. 1, pp. 23–34, 2010.

[8] A. H. Wu, R. G. Ziegler, P. L. Horn-Ross et al., "Tofu and risk of breast cancer in Asian-Americans," *Cancer Epidemiology Biomarkers and Prevention*, vol. 5, no. 11, pp. 901–906, 1996.

[9] P. This, A. De la Rochefordière, K. Clough, A. Fourquet, and H. Magdelenat, "Phytoestrogens after breast cancer," *Endocrine-Related Cancer*, vol. 8, no. 2, pp. 129–134, 2001.

[10] T. Usui, "Pharmaceutical prospects of phytoestrogens," *Endocrine Journal*, vol. 53, no. 1, pp. 7–20, 2006.

[11] J. B. Harborne and C. A. Williams, "Advances in flavonoid research since 1992," *Phytochemistry*, vol. 55, no. 6, pp. 481–504, 2000.

[12] F. Kohen, B. Gayer, T. Kulik et al., "Synthesis and evaluation of the antiproliferative activities of derivatives of carboxyalkyl isoflavones linked to N-t-Boc-hexylenediamine," *Journal of Medicinal Chemistry*, vol. 50, no. 25, pp. 6405–6410, 2007.

[13] H. Q. Li, H. M. Ge, Y. X. Chen et al., "Synthesis and cytotoxic evaluation of a series of genistein derivatives," *Chemistry and Biodiversity*, vol. 3, no. 4, pp. 463–472, 2006.

[14] L. N. Zhang, Z. P. Xiao, H. Ding et al., "Synthesis and cytotoxic evaluation of novel 7-O-modified genistein derivatives," *Chemistry and Biodiversity*, vol. 4, no. 2, pp. 248–255, 2007.

[15] K. Polkowski, J. Popiołkiewicz, P. Krzeczyński et al., "Cytostatic and cytotoxic activity of synthetic genistein glycosides against human cancer cell lines," *Cancer Letters*, vol. 203, no. 1, pp. 59–69, 2004.

[16] J. Popiołkiewicz, K. Polkowski, J. S. Skierski, and A. P. Mazurek, "In vitro toxicity evaluation in the development of new anticancer drugs—Genistein glycosides," *Cancer Letters*, vol. 229, no. 1, pp. 67–75, 2005.

[17] A. Rusin, J. Zawisza-Puchałka, K. Kujawa et al., "Synthetic conjugates of genistein affecting proliferation and mitosis of cancer cells," *Bioorganic and Medicinal Chemistry*, vol. 19, no. 1, pp. 295–305, 2011.

[18] D. E. Paterson, F. K. Griffin, M. L. Alcaraz, and R. J. K. Taylor, "A Ramberg-Bäcklund approach to the synthesis of C-glycosides, C-linked disaccharides, and C-glycosyl amino acids," *European Journal of Organic Chemistry*, no. 7, pp. 1323–1336, 2002.

[19] F. K. Griffin, D. E. Paterson, P. V. Murphy, and R. J. K. Taylor, "A new route to exo-glycals using the Ramberg-Bäcklund rearrangement," *European Journal of Organic Chemistry*, no. 7, pp. 1305–1322, 2002.

[20] G. Grynkiewicz, O. Zegrocka-Stendel, W. Pucko et al., "X-ray and C-13 CP MAS investigations of structure of two genistein derivatives," *Journal of Molecular Structure*, vol. 694, no. 1–3, pp. 121–129, 2004.

[21] M. B. Dinger and J. C. Mol, "Degradation of the first-generation Grubbs metathesis catalyst with primary alcohols, water, and oxygen. Formation and catalytic activity of ruthenium(II) monocarbonyl species," *Organometallics*, vol. 22, no. 5, pp. 1089–1095, 2003.

[22] D. F. Taber and K. J. Frankowski, "Grubbs' catalyst in paraffin: an air-stable preparation for alkene metathesis," *Journal of Organic Chemistry*, vol. 68, no. 15, pp. 6047–6048, 2003.

[23] M. Westhus, E. Gonthier, D. Brohm, and R. Breinbauer, "An efficient and inexpensive scavenger resin for Grubbs' catalyst," *Tetrahedron Letters*, vol. 45, no. 15, pp. 3141–3142, 2004.

[24] H. D. Maynard and R. H. Grubbs, "Purification technique for the removal of ruthenium from olefin metathesis reaction products," *Tetrahedron Letters*, vol. 40, no. 22, pp. 4137–4140, 1999.

[25] R. Roy, R. Dominique, and S. K. Das, "Extended alkenyl glycosides by ruthenium-catalyzed cross-metathesis reaction and application toward novel C-linked pseudodisaccharides," *Journal of Organic Chemistry*, vol. 64, no. 15, pp. 5408–5412, 1999.

[26] Y. J. Hu and R. Roy, "Cross-metathesis of N-alkenyl peptoids with O- or C-allyl glycosides," *Tetrahedron Letters*, vol. 40, no. 17, pp. 3305–3308, 1999.

[27] A. Kirschning and G. W. Chen, "Syntheses of spacer-linked neodisaccharides derived from L-Daunosamine," *Tetrahedron Letters*, vol. 40, no. 25, pp. 4665–4668, 1999.

[28] D. J. O'Leary, H. E. Blackwell, R. A. Washenfelder, and R. H. Grubbs, "A new method for cross-metathesis of terminal olefins," *Tetrahedron Letters*, vol. 39, no. 41, pp. 7427–7430, 1998.

[29] H. E. Blackwell, D. J. O'Leary, A. K. Chatterjee, R. A. Washenfelder, D. A. Bussmann, and R. H. Grubbs, "New approaches to olefin cross-metathesis," *Journal of the American Chemical Society*, vol. 122, no. 1, pp. 58–71, 2000.

[30] D. Forget-Champagne, M. Mondon, N. Fonteneau, and J. P. Gesson, "Selective cross-metathesis of 2-allylphenols with styrenes," *Tetrahedron Letters*, vol. 42, no. 41, pp. 7229–7231, 2001.

[31] A. K. Chatterjee, T. L. Choi, D. P. Sanders, and R. H. Grubbs, "A general model for selectivity in olefin cross metathesis," *Journal of the American Chemical Society*, vol. 125, no. 37, pp. 11360–11370, 2003.

[32] T. L. Choi, Choon Woo Lee, A. K. Chatterjee, and R. H. Grubbs, "Olefin metathesis involving ruthenium enoic carbene complexes," *Journal of the American Chemical Society*, vol. 123, no. 42, pp. 10417–10418, 2001.

[33] T. Velasco-Torrijos and P. V. Murphy, "Metathesis of structurally preorganized bivalent carbohydrates. Synthesis of macrocyclic and oligomeric scaffolds," *Organic Letters*, vol. 6, no. 22, pp. 3961–3964, 2004.

[34] M. H. D. Postema and J. L. Piper, "Cross-metathesis and ring-closing metathesis of olefinic monosaccharides," *Tetrahedron Letters*, vol. 43, no. 39, pp. 7095–7099, 2002.

[35] T. Ritter, A. Hejl, A. G. Wenzel, T. W. Funk, and R. H. Grubbs, "A standard system of characterization for olefin metathesis catalysts," *Organometallics*, vol. 25, no. 24, pp. 5740–5745, 2006.

[36] D. R. Anderson, T. Ung, G. Mkrtumyan, G. Bertrand, R. H. Grubbs, and Y. Schrodi, "Kinetic selectivity of olefin metathesis catalysts bearing cyclic (alkyl)(amino)carbenes," *Organometallics*, vol. 27, no. 4, pp. 563–566, 2008.

Hindawi Publishing Corporation
Journal of Chemistry
Volume 2013, Article ID 590512, 5 pages
http://dx.doi.org/10.1155/2013/590512

Research Article

Optimization of Extraction of Natural Pigment from Purple Sweet Potato by Response Surface Methodology and Its Stability

Jinwei Li,[1,2] Lianfu Zhang,[2] and Yuanfa Liu[2]

[1] *State Key Laboratory of Dairy Biotechnology, Technology Center, Bright Dairy & Food Co. Ltd., Shanghai 200436, China*
[2] *State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi 214122, China*

Correspondence should be addressed to Yuanfa Liu; ljw6688@hotmail.com

Received 8 November 2012; Revised 19 March 2013; Accepted 2 April 2013

Academic Editor: Mehmet Emin Duru

Purple sweet potato colour (PSPC) was a kind of natural pigment that attracted the general concern in recent years. In this paper, the response surface methodology was employed to optimize the extraction conditions of PSPC. The results showed that the extraction yield of purple colour was 11.6355 mg/g at the optimum extraction conditions of extraction temperature 60°C, extraction time 1 h, the ratio of solid to liquid ratio of 1 : 30, and acidified ethanol solution concentration 80%. Stability experiment showed that Fe^{3+} and Al^{3+} could increase the stability of PSPC, but Cu^{2+}, Zn^{2+}, and Pb^{2+} would decrease the stability of PSPC. Ascorbic acidified could significantly increase the stability of PSPC, and Na_2SO_3 would reduce the PSPC's stability.

1. Introduction

The colour of foods could affect the customer's decision on purchasing behavior by causing customer's direct attention on the sensory. However, the synthetic pigment had a negative impact on human's healthy. More and more attentions are paid to the natural pigment, which could be served as a functional component.

Anthocyanin is one of the most important natural pigments. Purple sweet potato is a plant which is rich in anthocyanin. The anthocyanin from purple sweet potato could be not only used as the natural pigment but also used as the functional compound due to its obvious antioxidant, antimutation, and antineoplastic activities [1–8]. In this paper, the objective was to optimize the extraction condition by response surface methodology and investigate its stability.

2. Materials and Methods

2.1. Materials. Fresh purple sweet potato was purchased from Tianheyuan agricultural incorporation in Suzhou.

2.2. Method

2.2.1. Pretreatment of Purple Sweet Potatoes. Purple sweet potatoes were washed and chopped into pieces, then they were dried in the oven at 50°C for 12 hours. Finally, they were smashed and kept in brown desiccator.

2.2.2. The Maximum Absorbance Wavelength of Purple Sweet Potatoes Pigment. A UV-Vis spectrophotometer (UV-2102PCS) was used to determine the maximum absorbance of purple sweet potato pigment. Purple sweet potato powder was weighted and extracted in acidified ethanol aqueous solution at 60°C for one hour. Then, it was centrifuged at 5000 r/min for 15 min. The pH of supernatant was adjusted to 2, and then the supernatant was scanned from 200 nm to 700 nm.

2.2.3. Optimization of Extraction of Purple Sweet Potato Pigment. A RSM was used to optimize the extraction conditions for purple sweet potato pigment. A multivariate study based on Box-Behnken design was chosen to evaluate effects of extraction parameters. The four independent variables were

Table 1: The coding schedule of response surface methodology.

Independent variable/unit	Symbol	Level		
		−1	0	1
Extraction temperature/°C	X_1	55	60	65
Time/min	X_2	50	60	70
Solid-liquid ratio/1 : X	X_3	25	30	35
Acidified ethanol aqueous solution concentration/%	X_4	75	80	85

extraction temperature (X_1), extraction time (X_2), solid-liquid ratio (X_3), and acidified ethanol aqueous solution concentration (X_4), and three levels of each independents were chosen for study. The coded values of the three independent variables were summarized in Table 1. The response value was the anthocyanin yield that was calculated from the absorption measured at 525 nm by a UV-Vis diode array spectrophotometer.

2.2.4. Experiment of Verification. 1.0000 g purple sweet potato powder was weighted and PSPC was extracted according to the conditions which were optimized by RSM, and the results were compared with the modeling value.

2.2.5. Stability of PSPC.

(1) Effect of Metal Ion on PSPC Stability. $CuSO_4$, $FeCl_3$, $AlCl_3$, $PbCl_2$, and $ZnSO_4$ were weighted and added in PSPC solutions with the Fe^{3+}, Cu^{2+}, Al^{3+}, Pb^{2+}, and Zn^{2+} concentration of 100 mg/L. The pH value was controlled at 2 and was kept away from light at room temperature. Measure its absorption at 0 h, 24 h, and 48 h.

(2) Effect of Food Additives on PSPC Stability. Na_2SO_3, ascorbic acid were weighted and added in PSPC solutions with the different concentration. The solutions were kept away from light at room temperature for 2 h and then measured its absorption at 525 nm.

3. Results and Discussion

3.1. Measurement of the Maximum Absorption Wavelength. As shown in Figure 1, there are three peaks at 280~290 nm, 320~340 nm, and 520~530 nm. The peak at 280~290 nm is the characteristic absorption peak of polyphenol, the peak at 320~340 nm is the characteristic absorption peak of organic acid, and the peak located at 520~530 nm is the characteristic absorption peak of anthocyanins. The peak at 520~530 nm is chosen as testing wavelength.

3.2. Effect of Extractant on the PSPC Yield. As shown in Figure 2, compared with the other extractant, acidified methanol has the highest PSPC yield, followed by acidified ethanol aqueous solution, 5% HCl. Considering its use on food, safety, and cost, the acidified ethanol aqueous solution is chosen as the appropriate extractant.

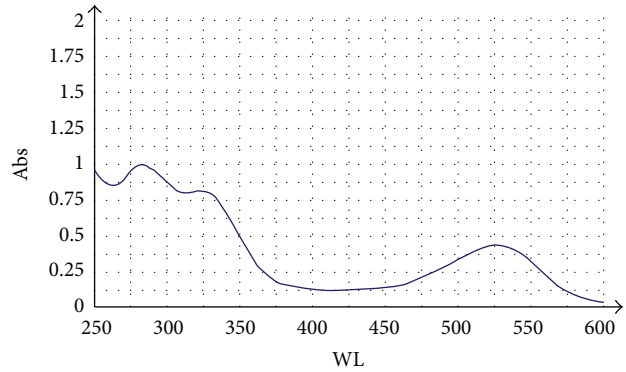


Figure 1: Full wavelength scan of PSPC.

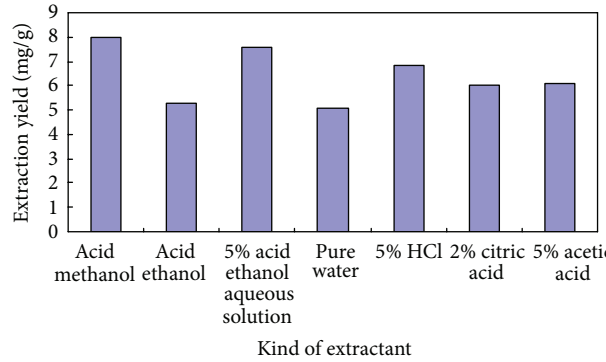


Figure 2: Effect of the kind of extractant on extraction ratio of PSPC.

3.3. Results of RSM Experiment. The results of RSM analysis of the variation of PSPC yield with extraction temperature (X_1), extraction time (X_2), solid-liquid ratio (X_3), and acidified ethanol aqueous solution concentration (X_4) are shown in Table 2. SAS multivariate regression program was used to analyze the data, and quadratic regression model of extraction temperature, time, solid-liquid ratio, and acidified ethanol aqueous solution concentration is as follows:

$$\begin{aligned} Y = {} & 24.93767 - 0.113417X_1 - 0.106833X_2 - 0.071X_3 \\ & - 0.50075X_4 - 1.490042X_1^2 - 0.0465X_1X_2 \\ & - 0.2835X_1X_3 + 0.64975X_1X_4 - 1.090667X_2^2 \\ & - 0.19725X_2X_3 - 0.09475X_2X_4 - 1.173917X_3^2 \\ & + 0.44675X_3X_4 - 0.902542X_4^2. \end{aligned} \quad (1)$$

3.4. Analysis of Variance (ANOVA) of Quadratic Regression Model. ANOVA of quadratic regression model (Table 3) demonstrated that the variables were adequately fitted to the regression equation (1), which were statistically acceptable at $P < 0.05$ level and adequate with satisfactory determination coefficients (R^2 of 0.9768 and R^2_{Adj} of 0.9468). The PSPC yield was significantly affected by linear term X_4, quadratic terms X_1^2, X_2^2, X_3^2, and X_4^2, and interactions terms of X_1 and X_3, X_1 and X_4, X_3, and X_4. According to the F value, the significance

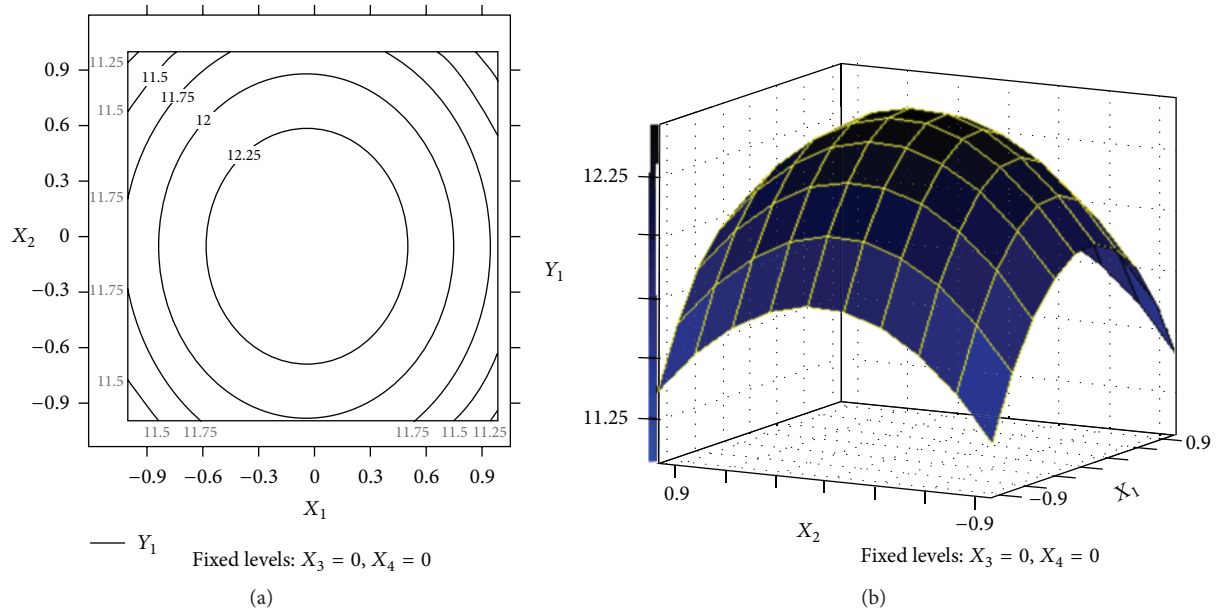


Figure 3: Contour plot and response surface diagram of effect between extraction temperature and time on PSPC.

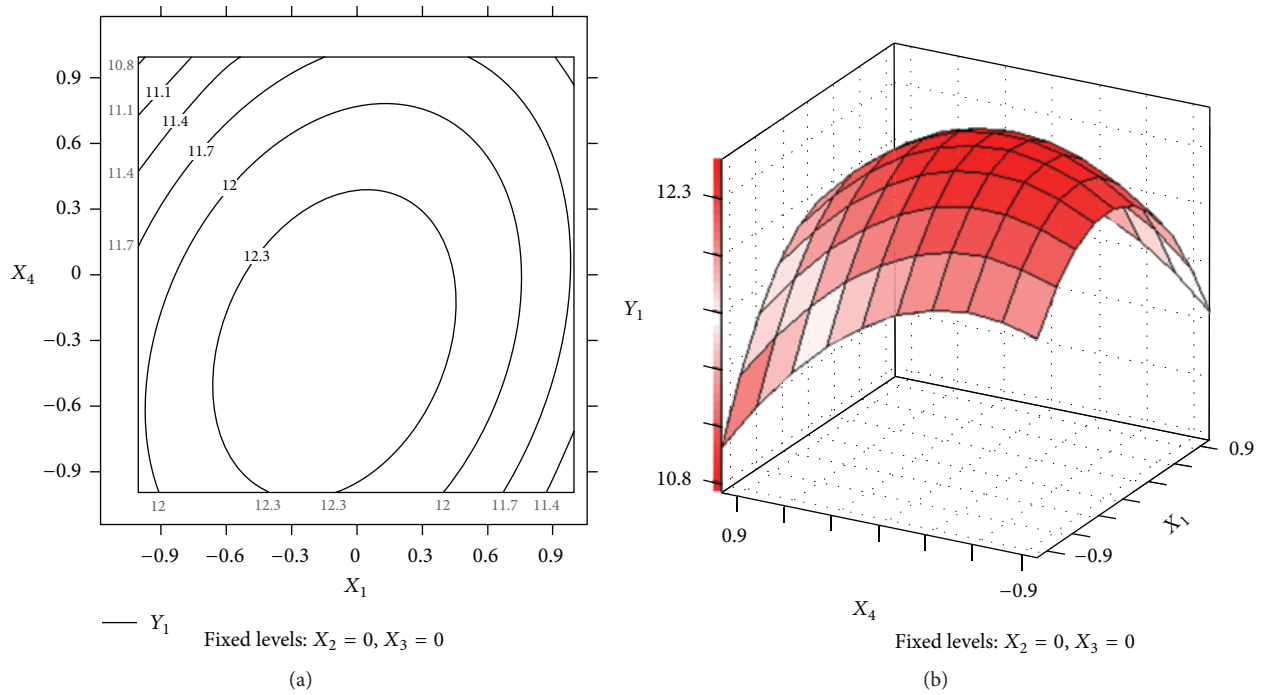


Figure 4: Contour plot and response surface diagram on effect between extraction temperature and concentration of acidified ethanol on PSPC.

orders of the parameters for the PSPC yield in the prediction model were $X_4 > X_1 > X_2 > X_3$.

3.5. Optimum Extraction Condition for PSPC. Three-dimensional diagram and contour plot made by full model of (1) were used to predict the relationships between the extraction temperature, extraction time, and PSPC yield (Figure 3). When the extraction temperature and extraction time increased, the PSPC yield increases firstly and then decreases. At the medium value of extraction condition of temperature and time, the PSPC has the higher extraction yield (Figure 3). The contour plot showed that the optimum extraction temperature is between 57.1 and 62.55°C and the optimum extraction time is between 56.6 and 62.95 min.

Three-dimensional diagram and contour plot were used to predict the relationships between the extraction

Table 2: Results of Box-Behnken of response surface methodology.

Test code	X_1	X_2	X_3	X_4	Y (mg/g)
1	−1	−1	0	0	11.3175
2	−1	1	0	0	11.1425
3	1	−1	0	0	11.3365
4	1	1	0	0	11.0685
5	0	0	−1	−1	11.9935
6	0	0	−1	1	10.9165
7	0	0	1	−1	11.5735
8	0	0	1	1	11.3900
9	−1	0	0	−1	11.8120
10	−1	0	0	1	10.7630
11	1	0	0	−1	11.1080
12	1	0	0	1	11.3585
13	0	−1	−1	0	11.3780
14	0	−1	1	0	11.3700
15	0	1	−1	0	11.4760
16	0	1	1	0	11.0735
17	−1	0	−1	0	11.3995
18	−1	0	1	0	11.0815
19	1	0	−1	0	10.8575
20	1	0	1	0	11.1065
21	0	−1	0	−1	11.6355
22	0	−1	0	1	11.2575
23	0	1	0	−1	11.7305
24	0	1	0	1	11.1630
25	0	0	0	0	12.4465
26	0	0	0	0	12.4800
27	0	0	0	0	12.4800

Table 3: Analysis of variance of regression model.

Source	DF	SS	MS	F	Pr > F
X_1	1	0.15436	0.15436	3.509213	0.085582
X_2	1	0.13696	0.13696	3.113648	0.103052
X_3	1	0.060492	0.060492	1.375222	0.263671
X_4	1	3.009007	3.009007	68.40659	0.0001
$X_1 \times X_1$	1	11.8412	11.8412	269.1971	0.0001
$X_1 \times X_2$	1	0.008649	0.008649	0.196626	0.665347
$X_1 \times X_3$	1	0.321489	0.321489	7.308713	0.019186
$X_1 \times X_4$	1	1.6887	1.6887	38.39082	0.0001
$X_2 \times X_2$	1	6.344287	6.344287	144.2307	0.0001
$X_2 \times X_3$	1	0.15563	0.15563	3.538089	0.084454
$X_2 \times X_4$	1	0.03591	0.03591	0.816382	0.38402
$X_3 \times X_3$	1	7.349762	7.349762	167.0891	0.0001
$X_3 \times X_4$	1	0.798342	0.798342	18.14947	0.001107
$X_4 \times X_4$	1	4.344434	4.344434	98.76612	0.0001
Model	14	22.27012	1.590723	36.16341	0.0001
Error	12	0.527845	0.043987		
Total	26	22.79797			

R^2 = 97.68% R^2_{Adj} = 94.68%.

Table 4: Effects of metallic ion on PSPC stability.

Time (h)	Absorbance value (A)					
	Fe^{3+}	Zn^{2+}	Cu^{2+}	Pb^{2+}	Al^{3+}	Blank
0	0.752	0.752	0.752	0.752	0.752	0.752
24	0.750	0.721	0.745	0.744	0.749	0.746
48	0.748	0.710	0.742	0.743	0.748	0.745

temperature and concentration of acidified ethanol solution and PSPC yield (Figure 4). When the extraction temperature and the concentration of acidified ethanol aqueous solution increased, the PSPC yield increased before the medium level and then decreases. The contour plot showed that the optimum extraction temperature is between 55 and 62°C and the optimum concentration of acidified ethanol aqueous solution is between 76.7% and 82.25%.

According to the results of the analysis of regression model, the optimum conditions for the extraction of PSPC are at the extraction temperature of 60°C, time of 1 h, solid-liquid ratio of 1 : 30, acidified ethanol aqueous solution concentration of 80%, and the extraction yield could reach 11.6355 mg/g.

3.6. Verification Experiment. The extraction experiments were carried out under the optimum conditions of extraction temperature 60°C, time 1 h, solid-liquid ratio 1 : 30, and acidified ethanol aqueous solution concentration of 80%. The real PSPC yield was 11.5276 mg/g, which was adjacent to the modeling value of 11.6355 mg/g with standard error of 0.93%. This result indicates that this regression model could be used to indicate the relationship between extraction condition and PSPC yield and to predict the extraction yield of PSPC.

3.7. Effect of Metal Ion on PSPC Stability. Effects of metal ion on PSPC stability is shown in Table 4. Compared with bank solution, the solution with Fe^{3+} or Al^{3+} has the higher absorbance value at 525 nm for 24 h and 48 h, which meant that Fe^{3+} or Al^{3+} contributed to the PSPC stability. However, the solution with Cu^{2+}, Zn^{2+}, or Pb^{2+} has the lower absorbance value at 525 nm for 24 h and 48 h, which meant that Cu^{2+}, Zn^{2+} or Pb^{2+} was against the PSPC stability.

3.8. Effect of Ascorbic Acid and Na_2SO_3 on PSPC Stability. Effects of ascorbic acid and Na_2SO_3 on PSPC stability is shown in Table 5. The absorbance value at 525 nm increased with the increase in the ascorbic acid concentration, which means that ascorbic acid contributed to the PSPC stability and could be used as copigment. The absorption value at 525 nm decreases with the increase in concentration of Na_2SO_3, which indicates that Na_2SO_3 has a bad effect on PSPC stability.

4. Conclusion

In the basis of RSM experiment, the optimum extraction conditions of PSPC are the extraction temperature of 60°C, extraction time of 1 h, solid-liquid ratio of 1 : 30, and acidified ethanol aqueous solution concentration of 80%, and the

Table 5: Effects of ascorbic acid and Na_2SO_3 on PSPC stability.

	Ascorbic acid concentration (mg/L)				
A	0	0.2	0.4	0.6	0.8
	0.721	0.732	0.758	0.792	0.799
	Concentration of Na_2SO_3 (mg/L)				
A	0	0.01	0.02	0.04	0.06
	0.698	0.657	0.642	0.638	0.632

extraction yield of PSPC is 11.6355 mg/g. Fe^{3+} and Al^{3+} can contribute the stability of PSPC, but Cu^{2+}, Zn^{2+}, and Pb^{2+} would decrease the stability of PSPC. Ascorbic acid can significantly increase the stability of PSPC which can be used as copigment, and Na_2SO_3 would have bad effect on the stability of PSPC.

Acknowledgments

This work was supported by the Research Fund of National 12th Five-Year Plan of China (2011AA100806-3), the National Natural Science Foundation of China (31171703 and 31101361), Industry-Academia Cooperation Innovation Fund Projects of Jiangsu Province (BY20120460), Fundamental Research Funds for the Central Universities (JUSRP211A30), and the Open Project Program of State Key Laboratory of Dairy Biotechnology, Bright Dairy & Food Co. Ltd. (SKLDB2011-002).

References

[1] Z. Yang, Y. Wang, and L. Gao, "Research progress on purple sweet potato colore," *Journal of Qingdao University (E&T)*, vol. 19, no. 2, pp. 32–36, 2004.

[2] Z. Fang and Y. Ni, "Research progress on physiological functions of anthocyanins," *Guangzhou Food Science and Technology*, vol. 17, no. 3, pp. 60–62, 2001.

[3] G. Lu, X. Lan, X. Lou et al., "Study on physical and chemical properties of red pigments from purple sweet potato (PSP)," *Journal of Zhejiang Agricultural University*, vol. 22, no. 3, pp. 308–311, 1996.

[4] Y. Liu, L. Zhu, Z. Han et al., "Anti-oxidative effect of Ipomoea Batatas Poir Cv anthocyanins on mice irradiated by 60Co gamma ray," *Acta Acadimae Qingdao Umvesitatis*, vol. 41, no. 1, pp. 46–51, 2005.

[5] X. Meng, X. Cu, F. Wang et al., "In vivo antioxidant activity of sweet -potato anthocyanins in aged mice," *Medical Journal of Qilu*, vol. 20, no. 5, pp. 394–399, 2005.

[6] W. Han, *Extraction, Composition Analasis, Stability and Antioxidant Activity of Anthocyanins from Purple Sweet Potato*, Nanjing Agriculture University, 2008.

[7] Z. Wang, *Study on the Technology Conditions of Extraction and Purification and Property of Purple Sweetpotato Color*, Southwest University, 2010.

[8] J. Li, *Study of the Extraction, Purification by Membrane Technology and Stability of Anthocyanin Pigment from Purple Sweet Potato*, Nanchang University, 2007.

Hindawi Publishing Corporation
Journal of Chemistry
Volume 2013, Article ID 783058, 9 pages
http://dx.doi.org/10.1155/2013/783058

Research Article

Mechanism of Selective Inhibition of Yohimbine and Its Derivatives in Adrenoceptor $\alpha 2$ Subtypes

Liu Hai-Bo,[1] Peng Yong,[1] Huang Lu-qi,[2] Xu Jun,[3] and Xiao Pei-Gen[1]

[1] *Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences, Beijing 100193, China*
[2] *Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences, Beijing 100700, China*
[3] *School of Pharmaceutical Sciences, Sun Yat-sen University, Guangzhou 510006, China*

Correspondence should be addressed to Peng Yong; ypeng@implad.ac.cn

Received 12 November 2012; Revised 22 March 2013; Accepted 2 April 2013

Academic Editor: Mehmet Emin Duru

Some natural alkaloids from medicinal plants, such as yohimbine and its derivatives, have been reported with adrenoceptor (AR) $\alpha 2$ subtypes inhibiting activity. In trying to address the possible mechanism of the action, a set of homology models of AR $\alpha 2$ was built based on MOE. After that, docking and molecular dynamics methods were used to investigate the binding modes of yohimbine and its 2 derivatives in the active pocket of adrenoceptor $\alpha 2$ subtype A, B, and C. The key interactions between the 3 ligands and the 3 receptors were mapped. Binding mode analysis presents a strong identity in the key residues in each subtype. Only a few differences play the key role in modulating selectivity of yohimbine and its derivatives. These results can guide the design of new selective AR $\alpha 2$ inhibitors.

1. Introduction

Adrenoceptor (AR) is a signal receptor of epinephrine and norepinephrine and is mainly distributed in myocardial, vascular, and nervous system. Till now, 9 AR subtypes have been reported. They are classified into subtypes $\alpha 1$, $\alpha 2$ and β according to the pharmacological functions and sequences. $\alpha 1$ receptors have subtype A, B, and D. $\alpha 2$ receptors have subtypes A, B, and C. β receptors have $\beta 1$, $\beta 2$, $\beta 3$ [1, 2].

Since discovered in 1948, AR family has always been one of the most important targets for drug discovery. Both the agonist and the antagonist on different AR subtypes can be leading compounds with pharmacological effects, particularly the significant cardiovascular effect. Looking for the candidate with high selectivity among the AR subtypes is the emphasis in drug development. The $\alpha 2$ ARs are widely distributed throughout the peripheral and CNS. Agonists acting at $\alpha 2$ ARs have analgesic properties following supraspinal [3], spinal [4], peripheral [5], and systemic administration [6].

Some natural products acting on the AR receptor have been found as the active ingredients of traditional Chinese medicine. All the compounds are alkaloids and are the AR blockers. According to the skeleton, the compounds can be divided into three categories: rauwolfia alkaloids, such as yohimbine; protoberberine, such as berberine; and aporphine, such as xylopine (Table 1).

Yohimbine (CAS: 146-48-5) was first isolated from bark of a West African tree *Pausinystalia yohimbe* (Rubiaceae) in 1945. The plant has a long history of human use as a stimulant and aphrodisiac, especially as a herbal supplement to improve erectile function. To date, Yohimbine was also found in *Alchornea floribunda* Müll. Arg. (Euphorbiaceae) [14], *Rauvolfia vomitoria*, *Rauwolfia nitida*, *Rauwolfia serpentine*, *Rauvolfia yunnanensis*, and others [15].

The pharmacological research on Yohimbine has been started since 1949 [16]. The reported activities include antidiuretic, antiadrenaline, mydriatic, serotonin antagonist, and others. The most remarkable activity is selective inhibition on ARs, high affinity for the AR $\alpha 2$ subtypes, and moderate affinity for the $\alpha 1$ subtypes. Binding affinities among the AR $\alpha 2$ subtypes are $\alpha 2C$ (0.88 nM) > $\alpha 2A$ (1.4 nM) > $\alpha 2B$ (7.1 nM) [13, 17, 18]. Because of the high selectivity on AR subtypes, Yohimbine attracted more attention [11, 12]. Lots of pharmacological data have been reported, while the mechanism of the action is still unclear.

Table 1: The alkaloids with AR blocking activities.

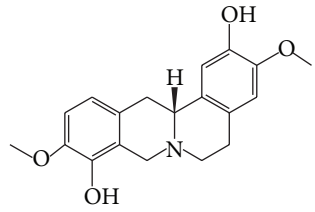
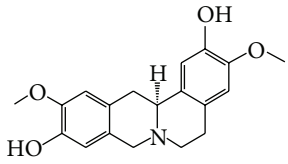
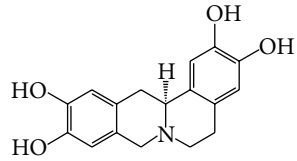
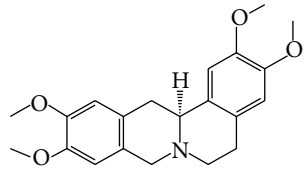
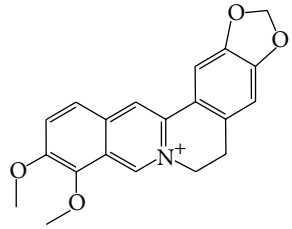
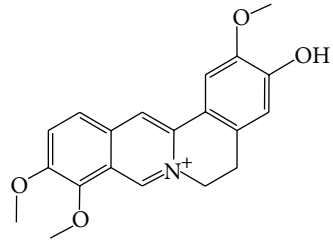
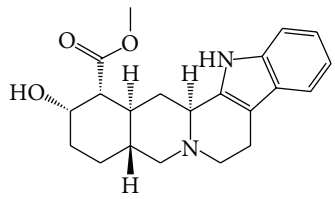
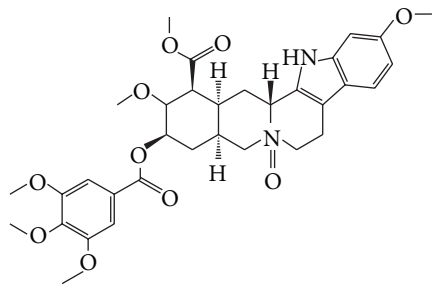
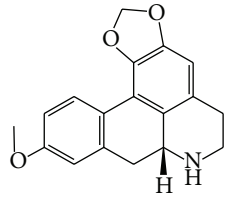
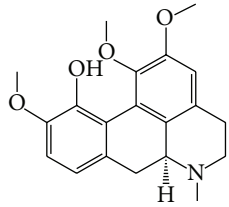
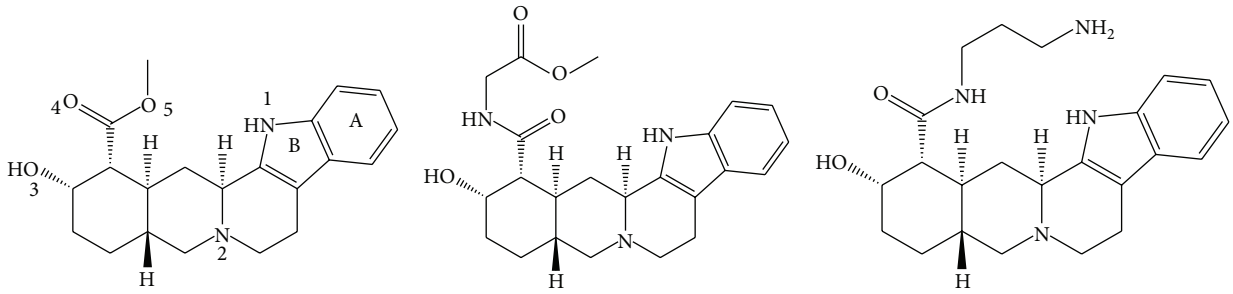
Name	Structure	Activities on ARs
(−)-Discretamine		α1D-AR blocking(rat) [7–9] Selectivity among various α1-AR subtypes(rat): α1A : α1B : α1D = 0.04 : 0.07 : 1.0
Govadine		Selective α1-AR antagonist, $\alpha 1 > \alpha 2 > \beta$ [10]
THB[1]		
Xylopinine		α-Receptor blocker
Berberine		α1, α2 Anatagonist; analgesic; antihypertensive; hypnotic; hypoglycemic; increases tolerance to anoxia; local anesthetic; reduces intraocular pressure (rabbit); vasodilator, vascular smooth muscle relaxant; smooth muscle stimulant
Jatrorrhizine		Antiarrhythmic; bidirectional action to heart (frog heart in vitro, inhibits first and then stimulates); antihypertensive; inhibits adrenaline(anesthetic rbt, iv); sedative(animal model); treatment of myocardial infarction (in coronary artery-ligated rbt)
Yohimbine		Antidiuretic; antiadrenaline; mydriatic; serotonin antagonist High affinity for the α2-AR, moderate affinity for the α1-AR Higher binding affinities at the α2C versus α2A and α2B [11] α2A/α2B = 4.8 [12] Binding affinities α2C (0.88 nM) > α2A (1.4 nM) > α2B (7.1 nM) [13]
Renoxidine		Antiadrenergic; anticonvulsant; antineoplastic; sedative; antihypertensive

Table 1: Continued.

Name	Structure	Activities on ARs
Xylopine		α1-AR blocker; analgesic; CNS depressant; platelet aggregation inhibitor
Isocorydine		Adrenergic antagonist; antiarrhythmic (animal model); increases coronary flow and cerebral blood flow

[1](+/−)-2,3,10,11-Tetrahydroxy tetrahydroprotoberberine HBr.



Yohimbine (a) Yohimbine monoglycine ester (YME) (b) Yohimbine alkyl amine (YAA) (c)

Figure 1: Yohimbine and its 2 derivatives.

In this paper, three-dimensional homology models were built for each AR α2 subtype. By using molecule stimulation method, the binding modes of Yohimbine, Yohimbine monoglycine ester (YME), and Yohimbine alkyl amine (YAA) were analyzed (Figure 1). The mechanism of selectivity and the key residues were investigated.

2. Data and Methods

2.1. Homo Modeling. The AR receptor belongs to heterotrimeric guanine nucleotide-binding protein coupled receptors (GPCRs). The proteins in GPCR superfamily usually have low sequence identities but with high structure similarities. The binding sites usually locate among the seven transmembrane (TM) helices; together with three extracellular loops. GPCR receptors play important roles in drug discovery [19]. Due to the hardness in the crystallization of GPCRs, homology modeling is a main approach for the structure-based drug design of GPCRs [20].

The secondary structure assignments are annotated with color-coded bars above the sequences. Helixes are in red, strands are in yellow, 1–4 turns are in blue, and 1–5 turns are in green. The conserved residues were marked with dark color.

To date, our understanding of GPCR structure is mainly based on the high-resolution crystal protein structure. Computational modeling has also predicted that GPCRs share a seven membrane-spanning a-helix topology as a common structural property. Likewise, mutagenesis studies have tentatively identified individual transmembrane domains with specific roles in signal transduction function that are conserved throughout the GPCR family. Since 2007, several Homo AR β-subtypes were reported [21–24]. Among the crystal data, 2RH1, a complex of β2 subtype with agonist carazolol, is an ideal template for the modeling of AR family [25, 26].

The alignment errors increase rapidly when the sequence identity of two proteins is less than 30% [27]. For GPCR homology, the sequence identity is usually less than 25% [20]. Therefore, automated homology modeling of GPCRs is likely to result in more errors [28]. The errors in alignments cannot be justified by energy optimizations, molecular dynamics, or distance geometry refinements. The alignments become critical for the quality of the homology model. Multiple sequence alignments are adopted in order to enhance the alignment quality. We have used 8 AR subtypes in the sequence alignment. As shown in Figure 2, all the subtypes keep a high

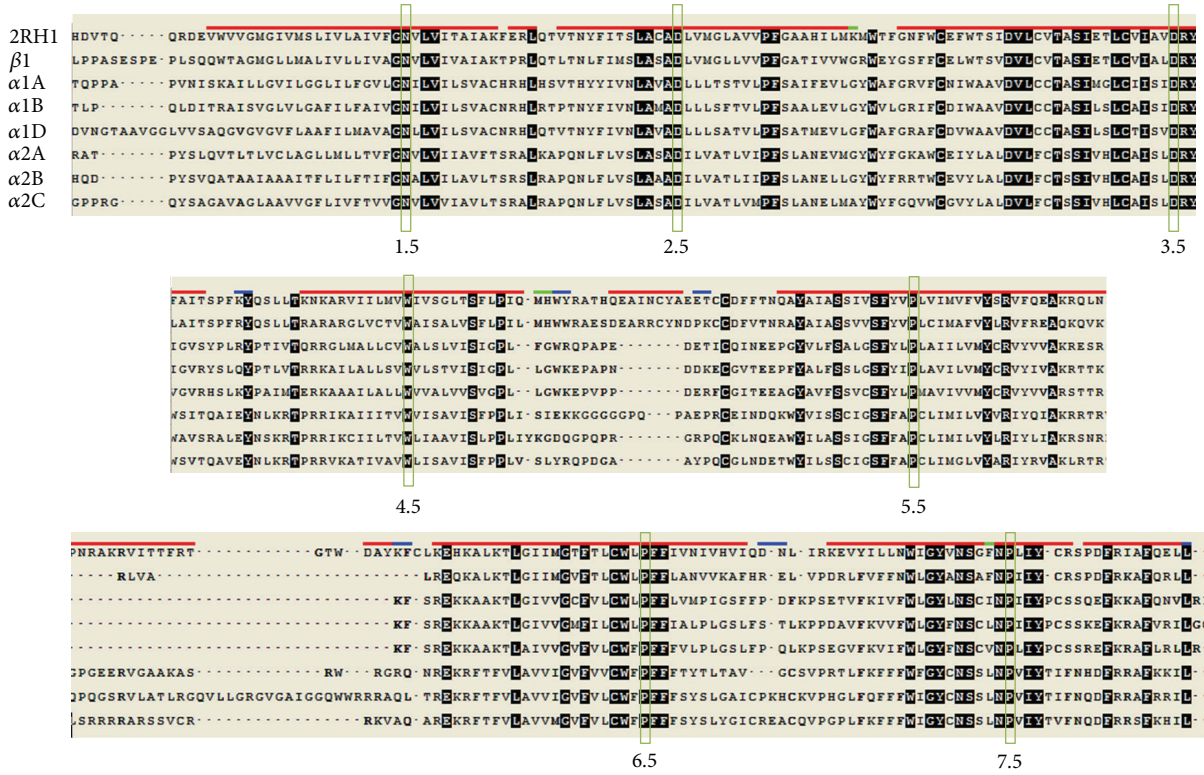


Figure 2: Alignment of the 8 AR subtypes.

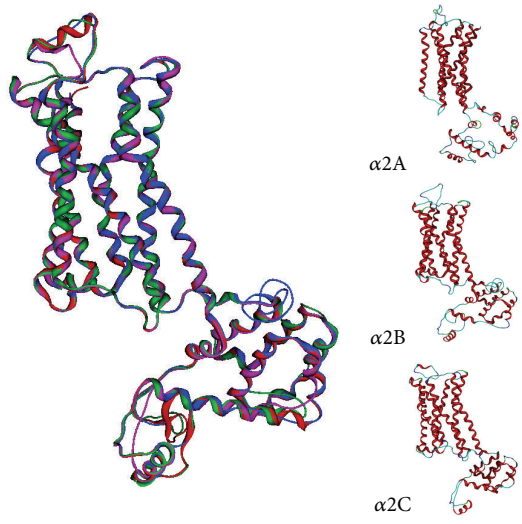


Figure 3: The homology models of three $\alpha 2$ subtypes. The left is the template superposed by the three homology models, 2RH1: red, $\alpha 2$A: green, $\alpha 2$B: blue, and $\alpha 2$C: purple.

identity in the TM helices. For the binding site is exactly constituted by the TMs, the homo model may be of high reliability to estimate the interaction between the ligand and AR.

Homo models of AR $\alpha 2$A, B and C were constructed with the homology modeling module of the molecular operating environment (MOE) [29]. The AMBER99 force field was applied to the homology modeling. The carboxyl-terminal and amino-terminal modeling was disabled. The scoring method was GB/VI [30]. Ten models were built by MOE for each subtype, kept in a MOE database, and ranked by the GB/VI scores. The best model was kept for the following energy optimizing.

Partial charges for all atoms were calculated. Then, the energy was minimized by means of AMBER99 force field, and the ending condition of the calculation was the root mean square (RMS) of the two conformer energies being less than 0.1 kcal mol^{-1} Å^{-1}. Furthermore, molecular mechanics (MD) simulations were applied, in MOE. The process consisted of two steps.

(1) The MD simulations were performed with all Cα atoms being fixed. The temperature was increased from 0 K to 300 K within 60 ps (picoseconds); an NVT (constant volume/constant-temperature) molecular dynamic simulation of 100 ps at 300 K was carried out with a time-step length of 0.002 ps.

(2) The atoms in the TM helixes were set flexible for conformation changes. The MD experiments simulated conformation changes in 1 ns timescale. The final model was obtained by energy minimization till an RMS of 0.05 kcal mol^{-1} Å^{-1} was reached.

2.2. Docking. Docking experiments were carried out to explore the binding modes of three antagonists, Yohimbine,

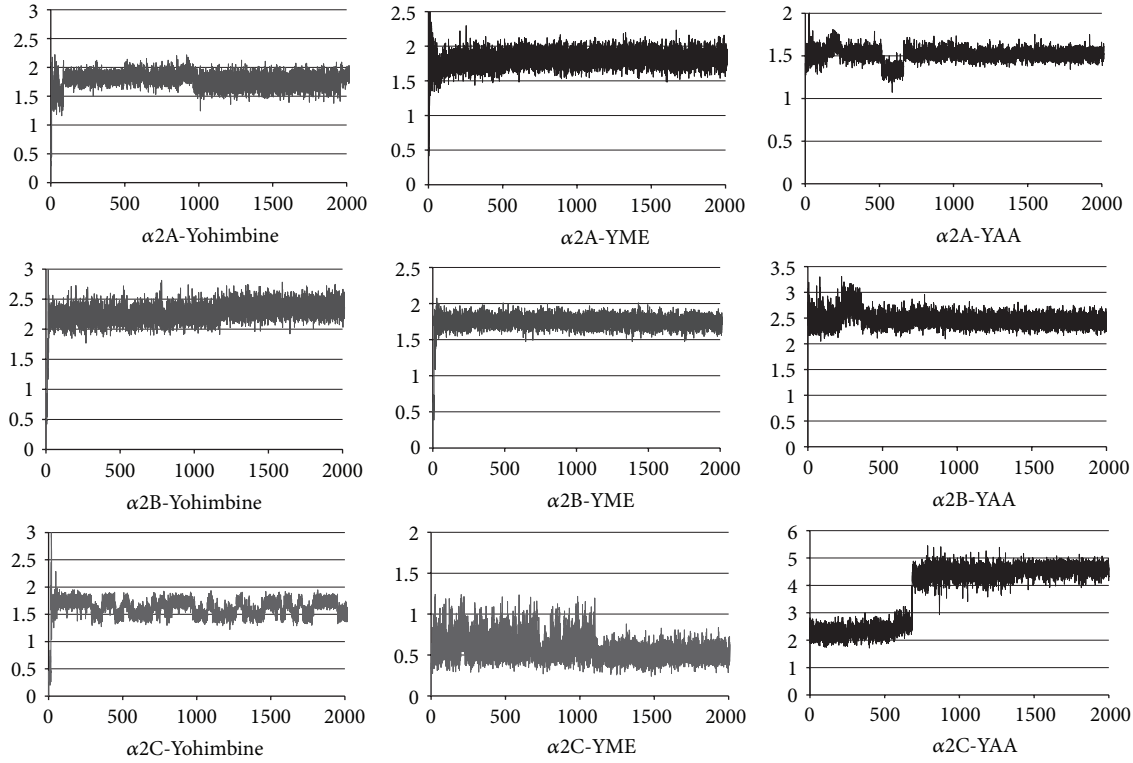


Figure 4: The RMSD fluctuation during MD simulation.

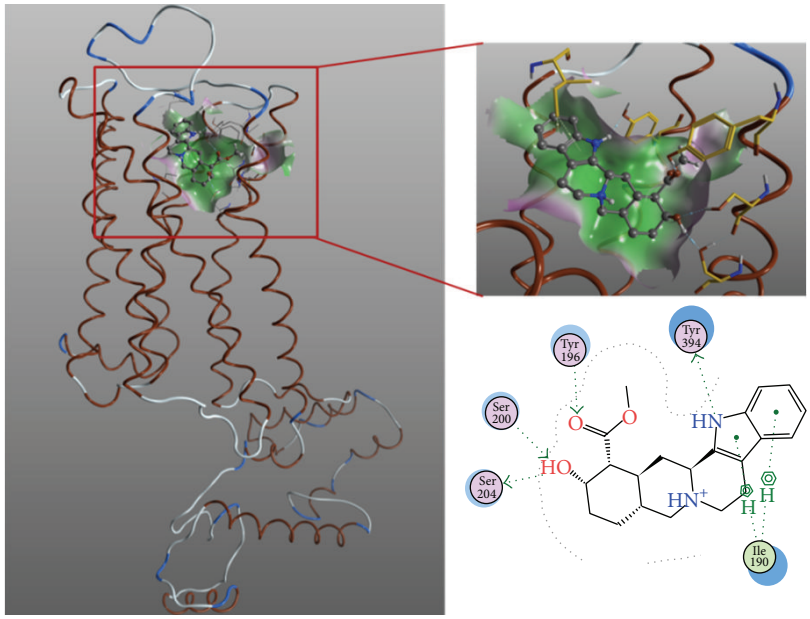


Figure 5: The binding mode of yohimbine with AR α2A.

YME, and YAA. All the ligands were built in MOE Builder. Their three-dimensional structures were energy minimized by MOE using MMFF94x force field. The initial poses were assigned by Flexible Alignment module of MOE, with the agonist carazolol in 2RH1 as the template. The 3 ligands were docked into 3 α2 subtypes, respectively. Totally 9 docking experiments were carried out.

Docking used the algorithm MOE Dock. The default settings were used. For each position, 700 iterations were generated to optimize the interactions at a location. A database

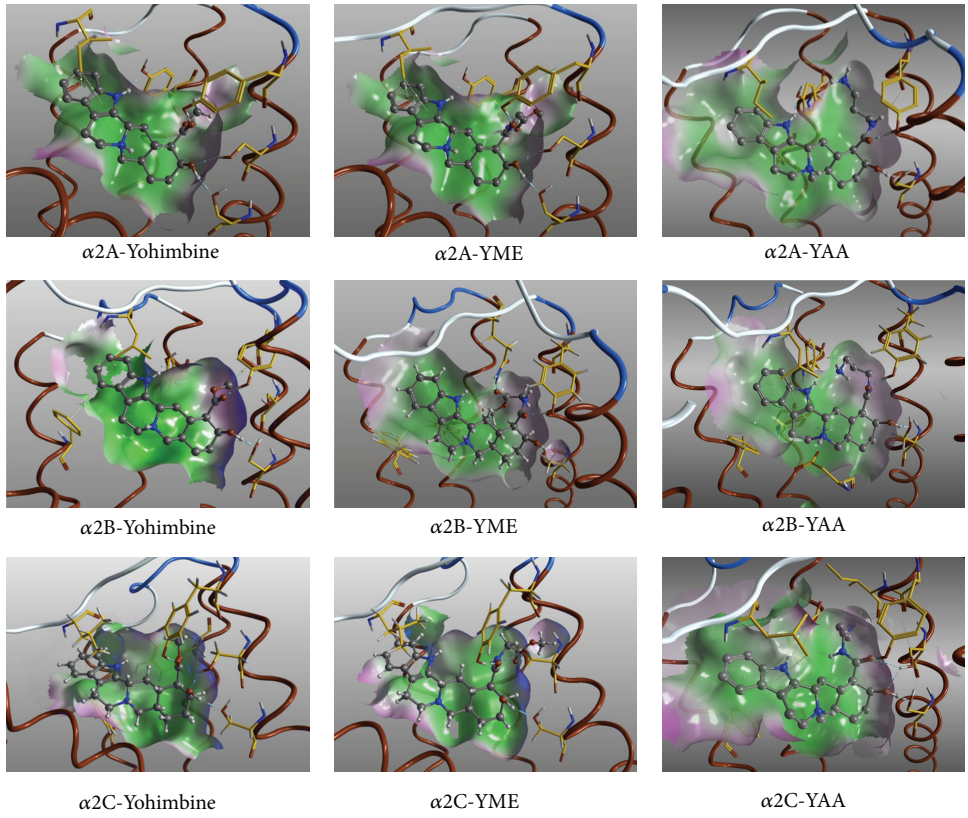


Figure 6: The pose of 3 ligands in the binding pocket of 3 subtypes.

of 25 complexes for each receptor with each ligand was generated. MMFF94 force field was used. Interactive forces between the ligand and the receptor include hydrogen bonding, aromatic interactions, and hydrophobic interactions. Of these, hydrogen bonding is the strongest interaction followed by aromatic interactions and last hydrophobic interactions. Therefore, the best complex was judged to be the complex with the greatest number of hydrogen bonds to side chain atoms, aromatic interactions, and placement in the helical bundle. No explicit water molecules were added during docking simulation. Solvation and entropic effects were not taken into account either.

The top 3 reasonable binding modes were picked, based upon compatibility with the reported mutation results and binding data, as well as energetic considerations, from the 10 resulting receptor-ligand complexes. A further refinement was done in MOE. The residues within 6 Å from the ligand were set to be flexible. Energy minimization was carried out by conjugated gradient minimization with the MMFF94x force field, until an RMSD of 0.1 kcal mol^{-1} $Å^{-1}$ was reached.

2.3. Dynamic Simulations on Docking Results. The top 3 complexes for each ligand and receptor were then subjected to two-stage molecular dynamics simulations. The simulation was performed for 1 fs time step with 200 ps of gradually heating phase from 0 K to 300 K, followed by 2000 ps equilibrium (data collection) phase. The output databases contained 11000 entries collected during the equilibrium phase. The simulation used NVT parameters (holding constant moles, volume, and temperature) and calculated potential energy (U in kcal mol^{-1}), temperature (T in kelvins), pressure (P in Kpa), total energy (E, kinetic and potential in kcal mol^{-1}), and enthalpy (H, $E + PV$ in kcal mol^{-1}). The complex with the least RMSD fluctuation was considered as the best one.

3. Result and Discussion

Figure 3 shows the template protein 2RH1 and the three established homology models. There are little structural differences among the template and the models in the 7 TM helices. The three-dimensional structure of binding pocket, surrounded by TM3, TM5, TM6, and eLP2, is especially close to each other among the 3 models. Because of the lack of conserved residues in loop domain, there are much more difference in the 3 endocellular loops and 3 extracellular loops. Since the active pocket is located in the upper half of the TMs, the differences of the loops do not affect the study on the binding mode of ligands. Therefore, the model can be used for the following molecular docking studies.

The 3 ligands have been docked to the homology structures of the 3 AR subtypes A, B, and C, respectively. For each combination, the most reasonable result was chosen as the start conformation for a further molecule dynamics optimization. The RMSD trajectories generated from the MD

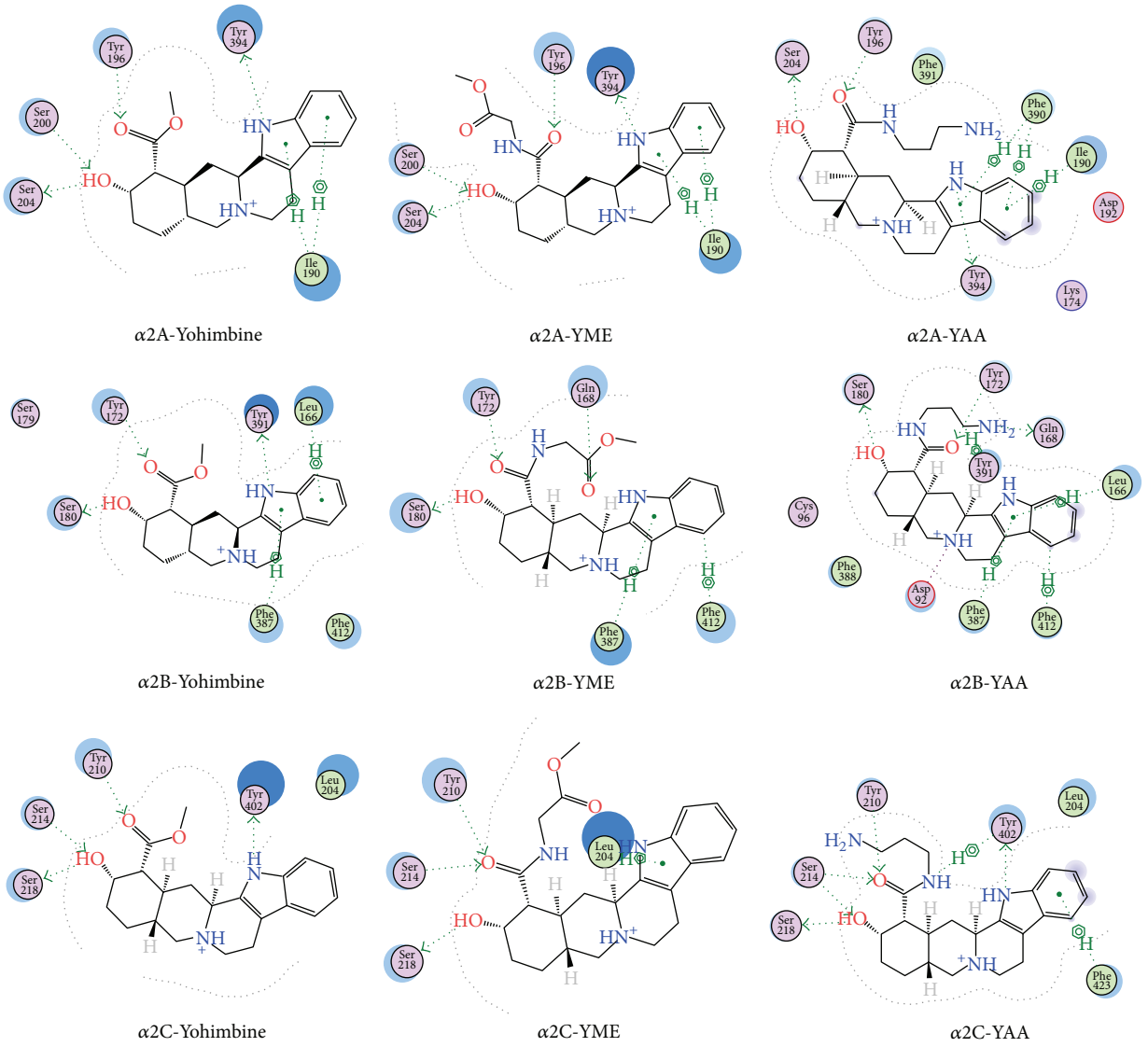


FIGURE 7: The binding modes of the 3 antagonists to 3 subtypes. Blue arrows represent hydrogen bonds formed with backbone atoms; green arrows represent hydrogen bonds formed with side-chain atoms.

simulation were shown in Figure 4. The final model was obtained by averaging the structures from the 1.5~2 ns trajectories of MD simulations. The model energy was minimized till an RMS of 0.05 kcal mol^{-1} $Å^{-1}$ was reached.

Figure 5 shows the binding mode of the Yohimbine in the α2A three-dimensional structure. The 3 ligands' 9 binding modes are depicted in Figures 6 and 7. The three-dimensional views are listed in Figure 6 and the ligand interaction maps are listed in the Figure 7. All the antagonists have the same orientation. The hydrophobic ends point to the opening of the pocket, and interact, through arene-H interaction, with Phe, Leu, or Ile residues on TM6 and eLP3. The polar ends face the bottom of the pocket; hydrogen bonds are formed with Tyr5.38, Ser5.42, and Ser5.46. The "hot" residues were identified by summarizing the interactions between the 3 ligands and the 3 α2 subtypes by 2D contact diagram of MOE (Figure 7, Table 2).

Table 2 summarized the interaction, hotspot residues, binding free energy, and reported pKi [11]. As can be seen, the binding mode of yohimbine is very close to that of YME and YAA. The interaction between the 3 ligands and the AR2 receptors mainly includes 4 aspects: (a) hydrogen bonds between oxygen atom 3 and 4 with polar residues Y5.38 and S5.46; (b) nitrogen atom 1 as a hydrogen bond donor formed a hydrogen bond with Y6.55; (c) ring A and the ring B contact with the aromatic/aliphatic residues, such as F6.51, I/L5.32, by arene-H interaction; (d) S5.42 may account for the selective inhibition of yohimbine and its derivatives.

As common structural characteristics through the three AR α2 subtypes, Y5.38, S5.42, S5.46, I/L5.32, and F6.51 are "hot" residues, the most interactive with the ligands (Table 2). The hydrogen bond interactions are the main contributors to binding affinities, which mainly located at the bottom of the AR binding pocket. The aromatic/aliphatic interactions

Table 2: Summary on the interactions between 3 ligands and the 3 α2 subtypes.

ligands	ARs	Hydrogen bond					Arene-H/arene-arene		E (kJ · mol^{-1})	pKi (μM)
		Y5.38	S5.42	S5.46	Y6.55	Others	F6.51	Others		
Yoh.	α2A	O-4	O-3/4	O-3	N-1			Ring-A/B-I5.32	−80.95	8.75
Yoh.	α2B	O-4		O-3	N-1		Ring-B	Ring-A-L5.32	−61.37	8.14
Yoh.	α2C	O-4	O-3/4	O-3	N-1			Ring-A/B-L5.32	−94.06	9.01
YME	α2A	O-4		O-3	N-1		Ring-B	Ring-A-I5.32	−83.16	7.47
YME	α2B	O-4		O-3			Ring-B	Ring-A/B-F412	−81.11	7.44
YME	α2C	O-4	O-3/4	O-3			Ring-B	Ring-A/B-L5.32	−85.24	8.07
YAA	α2A	O-4		O-3	N-1		Ring-A/B	Ring-A-I5.32	−77.26	6.64
YAA	α2B	O-4		O-3		NH2-D3.32	Ring-A/B	Ring-A-L5.32	−75.50	5.64
YAA	α2C	O-4	O-3/4	O-3			Ring-A	Ring-B-L5.32	−79.29	7.50

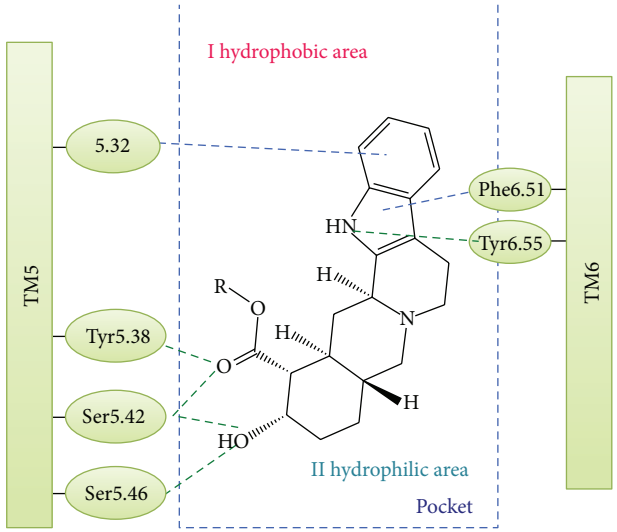


Figure 8: The 2 compartments binding modes for yohimbine and its derivatives.

are located at the opening of the pocket, close to the solvent. This area, the ligand tends to form arene-H bindings and hydrophobic interactions with "hot" residues, such as F6.51 and I/L5.32. Therefore, a good ligand should have at least one hydroxyl group at one end and two aromatic rings at the other end.

The root of the selectivity of the yohimbine and its derivatives may be the hydrogen bonds on S5.42. As shown in Table 2, the complexes with a hydrogen bond on S5.42 usually have a relatively higher pKi value. That also presents an explanation on yohimbine higher selectivity on AR α2C.

All the active pockets of AR α2 were formed by TM3, TM5, TM6, TM7, and eLP2. The pocket can be divided into two compartments (Figure 8). Compartment I is hydrophobic and close to the entrance of the pocket. Most residues in compartment I are aromatic/aliphatic, such as Phe, Leu, Ile and others. The most important of them are Phe6.51 and the Ile190 (α2A, eLP2), Leu166 (α2B, eLP2), and Leu204 (α2C, eLP2). Compartment II is a hydrophilic bottom of the binding pocket and has more hydrogen bonding opportunities, hosting "hot" residues as well: Tyr5.38, Ser5.42 and Ser5.46 on TM5.

Consequently, as to the structural characteristics of yohimbine and its derivatives, the 3 and 4 oxygen atoms and A, B rings (Figure 1) play the key role in the contact with ARs. The two oxygen atoms form "W" type 4 hydrogen bonds with Tyr5.38, Ser5.42, and Ser5.46, which all locate on TM5. The hydrogen bonds on Tyr5.38 and Ser5.46 are relatively stronger than those on Ser5.42. The two nitrogen atoms in the scaffold of yohimbine also interact with the residues on TM6 in some situations more or less. No. 1 nitrogen atom usually forms hydrogen bond with Tyr6.55; thus, it contributes more for the three ligands' affinity on AR α2. The hydrogen bonds between O-3 and S5.42 may contribute more in the formation of the selectivity of the ligands.

4. Conclusions

A set of homology models was built for AR α2 subtypes, based on multisequence alignments and secondary structure analyses. The 3 ligands were docked into 3 AR α2 subtype models. Molecular dynamics was carried to find the stable binding mode. The "hot" residues and the mechanism of the action are identified by comparing the 9 ligand-receptor complexes. These discoveries can be important for new AR α2 selective antagonist design.

Acknowledgments

The authors would like to thank Dr. Weiyu Lin for assistance in MOE SVL programing. The work in this paper was supported by the Ministry of Science and Technology of China, Special Project SB2007FY020.

References

[1] M. T. Piascik, E. E. Soltis, M. M. Piascik, and L. B. Macmillan, "α-adrenoceptors and vascular regulation: molecular, pharmacologic and clinical correlates," *Pharmacology and Therapeutics*, vol. 72, no. 3, pp. 215–241, 1996.

[2] B. Rozec and C. Gauthier, "β3-Adrenoceptors in the cardiovascular system: putative roles in human pathologies," *Pharmacology and Therapeutics*, vol. 111, no. 3, pp. 652–673, 2006.

[3] T. Z. Guo, J. Y. Jiang, A. E. Buttermann, and M. Maze, "Dexmedetomidine injection into the locus ceruleus produces

antinociception," *Anesthesiology*, vol. 84, no. 4, pp. 873–881, 1996.

[4] S. V. R. Reddy and T. L. Yaksh, "Spinal noradrenergic terminal system mediates antinociception," *Brain Research*, vol. 189, no. 2, pp. 391–401, 1980.

[5] K. D. Davis, R. D. Treede, S. N. Raja, R. A. Meyer, and J. N. Campbell, "Topical application of clonidine relieves hyperalgesia in patients with sympathetically maintained pain," *Pain*, vol. 47, no. 3, pp. 309–317, 1991.

[6] L. Paalzow, "Analgesia produced by clonidine in mice and rats," *Journal of Pharmacy and Pharmacology*, vol. 26, no. 5, pp. 361–363, 1974.

[7] F. N. Ko, J. H. Guh, S. M. Yu, Y. S. Hou, Y. C. Wu, and C. M. Teng, "(-)-Discretamine, a selective α(1D)-adrenoceptor antagonist, isolated from Fissistigma glaucescens," *The British Journal of Pharmacology*, vol. 112, no. 4, pp. 1174–1180, 1994.

[8] F. N. Ko, S. M. Yu, M. J. Su, Y. C. Wu, and C. M. Teng, "Pharmacological activity of (-)-discretamine, a novel vascular α-adrenoceptor and 5-hydroxytrypamine receptor antagonist, isolated from Fissistigma glaucescens," *The British Journal of Pharmacology*, vol. 110, no. 2, pp. 882–888, 1993.

[9] A. Carrieri, N. B. Centeno, J. Rodrigo, F. Sanz, and A. Carotti, "Theoretical evidence of a salt bridge disruption as the initiating process for the α1d-adrenergic receptor activation: a molecular dynamics and docking study," *Proteins*, vol. 43, no. 4, pp. 382–394, 2001.

[10] F. N. Ko, Y. L. Chang, C. M. Chen, and C. M. Teng, "(±)-govadine and (±)-THP, two tetrahydroprotoberberine alkaloids, as selective α1-adrenoceptor antagonists in vascular smooth muscle cells," *Journal of Pharmacy and Pharmacology*, vol. 48, no. 6, pp. 629–634, 1996.

[11] S. A. Bavadekar, G. Ma, S. M. Mustafa, B. M. Moore, D. D. Miller, and D. R. Feller, "Tethered yohimbine analogs as selective human α 2Cadrenergic receptor ligands," *Journal of Pharmacology and Experimental Therapeutics*, vol. 319, no. 2, pp. 739–748, 2006.

[12] W. Zheng, L. Lei, S. Lalchandani, G. Sun, D. R. Feller, and D. D. Miller, "Yohimbine dimers exhibiting binding selectivities for human α(2a)- versus α(2b)-adrenergic receptors," *Bioorganic and Medicinal Chemistry Letters*, vol. 10, no. 7, pp. 627–630, 2000.

[13] S. M. Mustafa, S. A. Bavadekar, G. Ma, B. M. Moore, D. R. Feller, and D. D. Miller, "Synthesis and biological studies of yohimbine derivatives on human α2C-adrenergic receptors," *Bioorganic and Medicinal Chemistry Letters*, vol. 15, no. 11, pp. 2758–2760, 2005.

[14] R. Paris and R. Goutarel, "African species of Alchornea; the presence of yohimbine in Alchornea floribunda (Euphorbiaceae).," *Annales pharmaceutiques françaises*, vol. 16, no. 1, pp. 15–20, 1958.

[15] J. J. Zhou, G. R. Xie, and X. J. Yan, *Chemical Components of Source Plants in Traditional Chinese Medicine*, Science Press, Beijing, China, 2009.

[16] C. M. Mac, C. I. Mc, and F. H. Shaw, "The action of yohimbine on excitation and propagation in nerve," *The Australian Journal of Experimental Biology and Medical Science*, vol. 27, part 1, pp. 115–122, 1949.

[17] M. J. Millan, A. Newman-Tancredi, V. Audinot et al., "Agonist and antagonist actions of yohimbine as compared to fluparoxan at alpha(2)-adrenergic receptors (AR)s, serotonin (5-HT)(1A), 5-HT(1B), 5-HT(1D) and dopamine D(2) and D(3) receptors. Significance for the modulation of frontocortical monoaminergic transmission and depressive states," *Synapse*, vol. 35, no. 2, pp. 79–95, 2000.

[18] D. D. Schwartz and T. P. Clark, "Selectivity of atipamezole, yohimbine and tolazoline for alpha-2 adrenergic receptor subtypes: implications for clinical reversal of alpha-2 adrenergic receptor mediated sedation in sheep," *Journal of Veterinary Pharmacology and Therapeutics*, vol. 21, no. 5, pp. 342–347, 1998.

[19] J. Saunders, "G-protein-coupled receptors in drug discovery," *Bioorganic & Medicinal Chemistry Letters*, vol. 15, article 3653, 2005.

[20] R. Heilker, M. Wolff, C. S. Tautermann, and M. Bieler, "G-protein-coupled receptor-focused drug discovery using a target class platform approach," *Drug Discovery Today*, vol. 14, no. 5-6, pp. 231–240, 2009.

[21] T. Warne, M. J. Serrano-Vega, J. G. Baker et al., "Structure of a β1-adrenergic G-protein-coupled receptor," *Nature*, vol. 454, no. 7203, pp. 486–491, 2008.

[22] S. G. F. Rasmussen, H. J. Choi, D. M. Rosenbaum et al., "Crystal structure of the human β2 adrenergic G-protein-coupled receptor," *Nature*, vol. 450, no. 7168, pp. 383–387, 2007.

[23] M. A. Hanson, V. Cherezov, M. T. Griffith et al., "A specific cholesterol binding site is established by the 2.8 A structure of the human beta2-adrenergic receptor," *Structure*, vol. 16, no. 6, pp. 897–905, 2008.

[24] M. P. Bokoch, Y. Zou, S. G. F. Rasmussen et al., "Ligand-specific regulation of the extracellular surface of a G-protein-coupled receptor," *Nature*, vol. 463, no. 7277, pp. 108–112, 2010.

[25] L. Ostopovici-Halip, R. Curpân, M. Mracec, and C. G. Bologa, "Structural determinants of the alpha2 adrenoceptor subtype selectivity," *Journal of Molecular Graphics and Modelling*, vol. 29, no. 8, pp. 1030–1038, 2011.

[26] V. Cherezov, D. M. Rosenbaum, M. A. Hanson et al., "High-resolution crystal structure of an engineered human β2-adrenergic G protein-coupled receptor," *Science*, vol. 318, no. 5854, pp. 1258–1265, 2007.

[27] D. Baker and A. Sali, "Protein structure prediction and structural genomics," *Science*, vol. 294, no. 5540, pp. 93–96, 2001.

[28] I. D. Pogozheva, M. J. Przydzial, and H. I. Mosberg, "Homology modeling of opioid receptor-ligand complexes using experimental constraints," *AAPS Journal*, vol. 7, no. 2, pp. E434–E448, 2005.

[29] "Molecular Operating Environment in, Chemical Computing Group Inc," Sherbrooke Street West, Suite 910, Montreal, H3A 2R7, Canada, 2007.

[30] P. Labute, "The generalized born/volume integral implicit solvent model: estimation of the free energy of hydration using London dispersion instead of atomic surface area," *Journal of Computational Chemistry*, vol. 29, no. 10, pp. 1693–1698, 2008.

Hindawi Publishing Corporation
Journal of Chemistry
Volume 2013, Article ID 409826, 10 pages
http://dx.doi.org/10.1155/2013/409826

Research Article

Secondary Metabolite Profile, Antioxidant Capacity, and Mosquito Repellent Activity of *Bixa orellana* from Brazilian Amazon Region

Annamaria Giorgi,[1,2] Pietro De Marinis,[1] Giuseppe Granelli,[1] Luca Maria Chiesa,[3] and Sara Panseri[3]

[1] *Department of Agricultural and Environmental Sciences-Production, Landscape, Agroenergy, Via Celoria 2, 20133 Milan, Italy*
[2] *Centre for Applied Studies in the Sustainable Management and Protection of the Mountain Environment, Ge.S.Di.Mont, Università degli Studi di Milano, Via Morino 8, Edolo, 25048 Brescia, Italy*
[3] *Department of Veterinary Science and Public Health, Università degli Studi di Milano, Via Celoria 2, 20133 Milan, Italy*

Correspondence should be addressed to Sara Panseri; sara.panseri@unimi.it

Received 12 November 2012; Revised 12 February 2013; Accepted 20 March 2013

Academic Editor: A. Hamid A. Hadi

The Brazilian flora was widely used as source of food and natural remedies to treat various diseases. *Bixa orellana* L. (Bixaceae), also known as annatto, urucù, or achiote, is a symbol for the Amazonian tribes that traditionally use its seeds as coloured ink to paint their bodies for religious ceremonies. The aim of this study was to investigate the volatile organic compounds (VOCs) profile of *B. orellana* fresh fruits (*in vivo* sampled), dried seeds, wood, bark, and leaves analyzed with Headspace solid-phase microextraction coupled with gas chromatography and mass spectrometry. A screening on phenolic content (the Folin-Ciocalteu assay) and antiradical activity (DPPH assay) of seeds was also conducted. In addition, the repellent properties of seed extracts against *Aedes aegypti* L. were investigated. Volatile compounds detected in *B. orellana* samples consisted mainly of sesquiterpenes, monoterpenes, and arenes: α-humulene is the major volatile compound present in seed extracts followed by D-germacrene, γ-elemene, and caryophyllene. *B. orellana* proved to be a good source of antioxidants. Preliminary data on repellency against *A. aegypti* of three different dried seed extracts (hexane, ethanol, and ethanol/water) indicated a significant skin protection activity. A protection of 90% and 73% for hexane and ethanol/water extracts was recorded.

1. Introduction

The Brazilian Amazon regions represent one of the world's richest pockets of biodiversity. Rich in endemic plants, tropical forests are known to host thousands of edible, medicinal and aromatic plant species [1]. These regions are also populated by numerous Indio tribes renowned for their traditional knowledge in using herbs as a source of food and natural remedies for different illnesses [2]. In 2006, Brazil's Ministry of Health established a policy regulating the use of medicinal plants and phytotherapeutic resources (Política Nacional de Plantas Medicinais e Fitoterápicos), aimed at improving healthcare and sustainable uses of biodiversity [3].

B. orellana L. (Bixaceae), a shrub native to Central and South America, also known as annatto, urucù, or achiote, is a symbol for the Amazonian tribes that traditionally use its seeds as coloured ink to paint their bodies for religious ceremonies [2]. Moreover, it is believed that the original Aztec chocolate beverage contained annatto seeds in addition to cocoa [4]. The term "annatto" in industrialized countries is commonly referred to *B. orellana* seed extract containing carotenoid-type pigments, widely used to dye an assortment of foods, textiles, and body care products [5]. Recently, *B. orellana* extracts have also been shown to be a viable option for commercial exploitation as a replacement for synthetic dyes and pigments in dyeing and finishing of leather [6]. Two carotenoids give annatto its unique red colour: bixin

and norbixin. Bixin (methyl (9-cis)-hydrogen-6,6-diapo-W,W-carotenedioate) is the main responsible for the orange-red colour of the seeds, and extracts of annatto represent approximately 80% of its total carotenoids [5, 7]. Therefore, *B. orellana* is a well-known commercial crop. The main commercial producers are tropical countries including Jamaica, Mexico, and the Philippines, with Peru, Brazil, and Kenya as the main sources of supply [8]. About 80% of the world production, which ranges between 10,000 and 11,000 tons of seeds per year, is utilized by the USA and Western Europe where it is further processed and used to impart the characteristic orange-yellow colour to butter, margarine, and cheese [9].

It is a matter of fact that almost all the organs of *B. orellana* are used in traditional medicine to obtain remedies to treat various diseases [10]. In Brazil, *B. orellana* leaves, roots, and seed extracts are popular as aphrodisiac medicines as well as a remedy to treat fevers, inflammatory conditions, and parasitic diseases. The entire plant is used against dysentery. Extracts of leaves, bark, and roots are reported to be antidotes for poisoning from *Manihot esculenta* (Crantz), *Jatropha curcas* L., and *Hura crepitans* L. [11]. A decoction of the leaves is used to stop vomiting and nausea, as a mild diuretic, to treat heartburn, prostate, urinary difficulties, and stomach problems [10].

Scientific evidences of *B. orellana* bioactivity are nowadays numerous, particularly regarding seed and leaf extracts. Anticonvulsant, antidiabetic, cardioprotective, and antimicrobial activities of different *B. orellana* extracts have been proved [12–14]. Fleischer et al. demonstrated the antimicrobial activity of *B. orellana* leaf and seed extracts against a range of Gram-positive and Gram-negative bacteria and fungi [15]. Moreover, some pharmacological studies have revealed that *B. orellana* extracts possess an antiprotozoal and anthelmintic effect [16]. Due to their broad antioxidant and antibacterial activities, polar extracts from *B. orellana* leaves and seeds have been recently proposed as alternative natural preservatives in food matrices [17]. Phytochemical investigations have revealed the presence of almost twenty-five types of chemical compounds from *B. orellana* different extracts, with the following major components: carbohydrates, carotenoids, steroids, proteins, flavonoids, terpenoids, phenolics, tannins, glycosides, alkaloids detected only in the leaf, and anthroquinones only in the seeds [15, 18].

Despite being widely used and studied, little is known about *B. orellana* volatile organic compounds (VOCs) composition, especially regarding the terpene profile from various plant organs. Moreover, even if *B. orellana* seed extracts claim to repel insects, little data for a clear validation is available. Therefore, the present research provides the first investigation on *B. orellana* chemical composition of different plant organs (fruits, leaves, bark, wood, and seeds) and the volatile profile sampled from living plants growing in an Amazonian Reserve (*in vivo* sampling) during an Italian-Brazilian cooperation project. In addition, a preliminary investigation on the repellent properties of seed extracts against *A. aegypti* L. was conducted.

2. Materials and Methods

2.1. Plant Samples. *B. orellana* plants growing in proximity of the Upper Rio Guamà Reserve located in the municipality of Capitao Poço, Parà State, Brazil (46°59′54.82″ O; 1°52′4.08″ S; 152 m. a.s.l.) were identified, and the volatile compounds emissions of fruits were *in vivo* sampled in February 2011 during a mission of an Italian-Brazilian cooperation project. Dry seeds, roots, leaves, bark, and wood were bought at Belem, at the Ver-o-Peso commercial district, from the most frequented herbalist shop (1°27′8.84″S; 48°30′8.99″O). Identification of the *in vivo* sampled plants and purchased materials was participatory and based on traditional knowledge of Indios Tembè, a tribe living in Itaputyr, a village in the upper Rio Guamà reserve [19, 20].

2.2. Standards and Chemicals. 2,2-diphenyl-1-picrylhydrazyl (DPPH), the Folin-Ciocalteu reagent, sodium carbonate, methanol, and ethanol were purchased from Sigma-Aldrich, Milan, Italy.

Volatile compounds used as references were purchased from Sigma-Aldrich-Fluka, from the General and Flavours and Fragrances catalogue, Milan, Italy.

2.3. Superfine Grinding (SFG). In order to obtain a representative plant sample a superfine powder was prepared from *B. Orellana*'s different organs (seeds, wood, bark, and leaves) using mechanical grinding activation in an energy intensive vibrational mill. Three g of dry plant material samples were ground in a high intensity planetary mill Retsch (model MM 400, Retsch, GmbH, Retsch-Allee, Haan), as previously reported [21]. The mill was vibrating at a frequency of 25 Hz for 4 min using two 50 mL jars with 20 mm stainless steel balls. Precooling of jars was carried out with liquid nitrogen in order to prevent the temperature from increasing during the grinding process. The speed differences between balls and jar resulted in the interaction of frictional and impact forces, releasing high dynamic energies. The interplay of all these forces resulted in the very effective energy input of planetary ball mills.

2.4. Seed Extracts Preparation. Three different *B. orellana* seed extracts were prepared to evaluate antioxidant capacity and phenolic content.

2.5. Traditional Infusion. Aliquots of 1 g from *B. orellana* seed powder were infused for 15 min with 100 mL of freshly boiled distilled water and cooled to room temperature. The infusion was then filtered using Whatman No. 4 filter paper in order to remove plant residues, and the aliquots were stored at +4°C overnight until the analysis.

2.6. Exhaustive Extraction (H_2O and EtOH). Aliquots of 0.3 g of B. *orellana* seed powder were subjected, respectively, to sequential extraction using 8 mL of distilled water, sonicated for 10 minutes, macerated for 1 h, and then sonicated with an ultrasonic cleaning bath (Branson 2510, 20 KHz, 120 W) for a further 30 min at room temperature. The procedure

was repeated eight times to obtain a final volume of 64 mL of extract. This similar procedure was repeated for new samples using 8 mL of EtOH solution. Extracts were then filtered using Whatman No. 4 filter paper, and the aliquots were stored at +4°C until further analysis.

2.7. 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Assay. Antioxidant capacity of the extracts was measured by the stable radical DPPH as previously reported [21]. Each extract was diluted with methanol to five different concentrations between 0.09 and 0.74 mg mL^{-1}. The DPPH concentration in methanol was 0.1 g L^{-1}. One mL of extract solution (sample) or methanol (control) was mixed with 1 mL of DPPH, and the absorption was recorded after 20 min in the dark, at 517 nm (UV/VIS spectrophotometer, model 7800, Jasco, Milan, Italy). Antioxidant capacity (AC) was calculated according to the following equation:

$$\text{AC } [\%] = 100 \times \frac{A_c - A_s}{A_c}, \quad (1)$$

where A_s is the absorbance of the sample and A_c is the absorbance of the control. The concentration of extracts causing a 50% decrease of DPPH solution absorbance (IC_{50}) was calculated by linear regression of the concentration-response plots, where the abscissa represented the concentration (mg mL^{-1}) of the extracts and the ordinate of the average percentage of AC from three separate determinations. The antioxidant capacity of all *B. orellana* extracts was expressed as $1/IC_{50}$ (mean value).

2.8. Determination of Total Phenol Content. The amount of total phenols in *B. orellana* extracts was determined using the Folin-Ciocalteu reagent according to the modified method of Slinkard and Singleton, 1977, using Gallic acid as a standard, as previously reported [21]. Distilled water (1550 μL) was combined and vortexed with 50 μL of sample (4.6 mg dry w/mL) and 100 μL of Folin-Ciocalteu's reagent. Then, 300 μL of Na_2CO_3 (20%) was added to reach a final concentration of 0.05 mg mL^{-1}; the mixture was vortexed. The absorbance of all samples was measured at 765 nm using a UV/VIS spectrophotometer (model 7800, Jasco, Milan, Italy) after incubation at 40°C for 30 min. Quantification was done on the basis of the standard curve of Gallic acid (solution of Gallic acid 10% EtOH, 0.25–10 μg/mL). The calibration equation for Gallic acid was $y = 2.5509x + 0.0096$, $r^2 = 0.9986$. The results were expressed as milligrams of gallic acid equivalents (GAE) per gram of dry weight. Measurements were taken in triplicate and expressed as mean value.

2.9. Seed Extracts for Mosquito Repellency Activity. To evaluate mosquito repellency activity of *B. orellana,* different extracts from powder seeds were prepared using a 5-step extraction/drying procedure (30 mL solvent for each step) using three different solvents: ethanol, ethanol/water (50 : 50 v/v), and hexane, as reported in Table 1. Dry residues were stored at 4°C until resuspension in 5 mL of solvent for skin application (Table 2).

Table 1: Different extraction conditions of *B. orellana* seed powder for mosquito repellency evaluation.

Extraction solvent	Sample weight (g)	Dried extract (g)
Hexane	2.026	0.091
EtOH	2.034	0.178
EtOH/H_2O (50 : 50)	2.002	0.569

Table 2: *B. orellana* seed extracts for mosquito repellency evaluation.

Extraction solvent	Resuspension solvent (5 mL)	Final solution (mg/mL)
Hexane	Acetone	18.2
EtOH	EtOH	35.6
EtOH/H_2O (50 : 50 v/v)	EtOH/H_2O (50 : 50 v/v)	113.8

2.10. Repellent Activity Test. The method of percentage of protection in relation to the dose was used [22]. Hundred blood-starved *Aedes aegypti* females (4–8 days old), reared at 25°C, 80% RH, and an L:D cycle of 12:12 hours, were kept in a net cage (45 × 30 × 45 cm). Before each test, the untreated forearm (control) of some experimenters was exposed to mosquitoes in the test chamber. Once the experimenters observed five mosquito landings on the untreated arm, they removed their arm from the chamber. The arms of the test person were cleaned with ethanol, washed with unscented neutral soap, thoroughly rinsed, and allowed to dry 10 min before extracts application. After air drying the arms of the tested person, only 25 cm^2 ventral side of the skin on each arm was treated and exposed, while the remaining area was covered by rubber gloves and apposite covering bracelets. The different extracts of *B. orellana* seeds (at three different concentrations each) were applied until complete covertures of the skin in three different trials. The control and treated arms were introduced serially into the cage. The number of landings was counted over 3 min at 11:00 a.m, at 2:00 p.m and at 6:00 p.m to evaluate differences in mosquito appetite. The experiment was conducted by 3 human volunteer testers and replicated three times at each concentration. All experiments were carried out at a temperature of 27 ± 1°C and RH 75 ± 5% under laboratory conditions. No skin irritation from the tested seed extracts was observed.

The percentage of protection was calculated by using the formula:

$$\text{protection } \% = \frac{N^\circ\text{of landings control} - N^\circ\text{of landings treated}}{N^\circ\text{of landings control}} * 100. \quad (2)$$

2.11. Headspace Solid-Phase Microextraction (HS-SPME) Volatile Compounds Sampling from B. orellana Living Plants (In Vivo). B. orellana opened fruits were sampled *in vivo* in order to evaluate the VOCs fingerprint emitted by living plants. Each fruit was enclosed in a customised Teflon cage

(manufactured by SNK, Inc., Fullerton, CA: 11 × 21 cm) into which a manual SPME holder was inserted to extract the headspace. VOCs were collected using 50/30 μm Divinylbenzene/Carboxen/polydimethylsiloxane (CAR/PDMS/DVB) StableFlex fibre (Supelco; Bellefonte, PA). The fibre was exposed to the plant headspace for 8 h. Long sampling time were selected in order to obtain a comprehensive VOCs profile emitted by plants. Peaks originating from the cage (Teflon material) were also assessed by extracting a blank sample.

2.12. HS-SPME Volatile Compounds Sampling of Different Dried B. orellana Organs (Seeds, Wood, Bark, and Leaves). The HS-SPME extraction conditions were optimised in our previous study on the characterisation of *Achillea collina* VOCs (selection of SPME fibre, sample amount, and extraction time) [23]. Briefly, all samples were prepared by weighing exactly 1.00 g of *B. orellana* powdered samples of seeds, wood, bark, and leaves, respectively, in a 20 mL glass vial, fitted with cap equipped with silicon/PTFE septa (Supelco, Bellefonte, PA, USA), and by adding 1 mL of the internal standard solution (IS) in water (1,4-cineol, 1 μg/mL, CAS 470-67-7) to check the quality of the fibres.

At the end of the sample equilibration period (1 h), a conditioned (1.5 h at 280°C) 50/30 μm Divinylbenzene/Carboxen/polydimethylsiloxane(CAR/PDMS/DVB) StableFlex fibre (Supelco; Bellefonte, PA) was exposed to the headspace of the sample for extraction (3 h) by CombiPAL system injector auto-sampler (CTC analytics, Switzerland). Temperature of 30°C was selected as extraction temperature in order to prevent possible matrix alterations (oxidation of some compounds, particularly aldehydes). To keep a constant temperature during the analysis, the vials were maintained on a heater plate (CTC Analytics, Zwingen, Switzerland).

2.13. Gas Chromatography-Mass Spectrometry Analysis of VOCs. HS-SPME analysis was performed using a Trace GC Ultra (Thermo-Fisher Scientific; Waltham, MA, USA) Gas Chromatograph coupled to a quadruple Mass Spectrometer Trace DSQ (Thermo-Fisher Scientific; Waltham, MA, USA) and equipped with an Rtx-Wax column (30 m; 0.25 mm i.d.; 0.25 μm film thickness, Restek, USA). The oven temperature programme was from 35°C, hold 8 min, to 60°C at 4°C/min, then from 60°C to 160°C at 6°C/min, and finally from 160°C to 200°C at 20°C/min. Carry-over and peaks originating from the fibre were regularly assessed by running blank samples. After each analysis fibres were immediately thermally desorbed in the GC injector for 5 min at 250°C to prevent contamination. The injections were performed in splitless mode (5 min). The carrier gas, helium, at a constant flow of 1 mL/min. An *n*-Alkanes mixture (C_8–C_{22}, Sigma R 8769, Saint Louis, MO, USA) was run under the same chromatographic conditions as the samples to calculate the Kovats retention indices (KI) of the detected compounds [24]. The transfer line to the mass spectrometer was maintained at 230°C, and the ion source temperature was set at 250°C. The mass spectra were obtained by using a mass selective detector with the electronic impact at 70 eV, a multiplier voltage of 1456 V, and by collecting the data at the rate of 1 scan/s over the *m/z* range of 30–350. Compounds were identified by comparing the retention times of the chromatographic peaks with those of authentic compounds analyzed under the same conditions when available or by comparing the Kovats retention indices with the literature data. The identification of MS fragmentation patterns was performed either by the comparison with those of pure compounds or using the National Institute of Standards and Technology (NIST) MS spectral database. Volatile compounds measurements from each headspace of the plant extracts were carried out by peak area normalization (expressed in percentages). All analyses were done in triplicate.

Data is expressed as mean value and standard deviation.

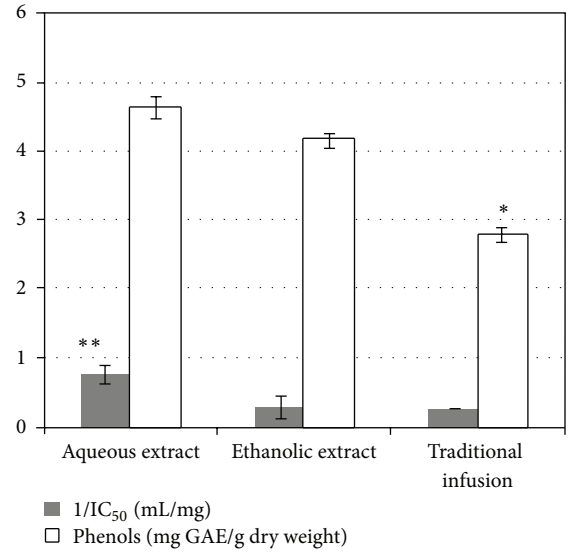


Figure 1: Antioxidant capacity (DPPH scavenging activity)[a] and total phenol content of different extracts of *B. orellana* seed samples (DW)[b]. Data expressed as mean ± standard deviation ($n = 3$). [a]: 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity expressed as inhibitory concentration ($1/IC_{50}$); **: significant $P < 0.05$. [b]: Phenol content expressed as mg GAE/g dry weight; *: significant $P < 0.05$.

3. Results and Discussion

3.1. Antioxidant Capacity and Total Phenol Content Analysis. Results of the antioxidant capacity and total phenol content (TPC) of all *B. orellana* seed extracts (traditional infusion, H_2O, and H_2O/ETOH) are presented in Figure 1. The free radical scavenging activity was evaluated by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method, which is regarded as an easy, reliable, sensitive, and rapid method. A review by Moon and Shibamoto also reported that almost 90% of antioxidant studies are conducted with this method [25]. We noticed that the DPPH scavenging activity was significantly greater for the seeds extracted with water than for extracts obtained with infusion and ethanol. It is well known that antioxidants react with DPPH free radical and convert it to the stable form. Therefore, *B. orellana* seeds extracted with H_2O seem

to contain a great amount of antioxidant compounds which may act as primary antioxidants able to react with free radicals as efficient hydrogen donors. A broad antioxidant activity of leaf and seed polar extracts has been recently proved using five different test systems by Viuda-Martos et al. [17]. Cardarelli et al. reported that the highest free radical scavenging capacity of annatto extracts was observed in the extract obtained with the most polar solvents, which also showed the highest phenol content [26].

In our conditions, the total phenol content resulted significantly higher for seed extracts obtained with H_2O than for infusion or H_2O/ETOH extraction.

This result clearly indicates that most of the phenols present in the plant are in a polar form. The TPC of *B. orellana* seed extracts, expressed as Gallic acid equivalent, ranged from 2.82 mg/g to 4.67 mg/g. Cardarelli et al. reported a lower TPC content that ranged between 0.30 and 1.84 mg of Gallic acid equivalent per gram of dry seeds (depending on the solvent used for extraction), while Chisté et al. recorded TPC values of 1.7 mg of Gallic acid equivalent per gram of wet seeds [26, 27]. The concentration and type of phenol substances present in the extracts are influenced by seasonal and environmental factors, for example, climate and soil type, genetic factors, and processing methods such as type of solvent used It is noteworthy that the water extractable phenol compounds present in *B. orellana* dry seeds are higher than that of green beans, tomatoes, green cabbage, green collard, and some green leafy vegetables. Bioactive phenols are very interesting as antioxidants because of their natural origin and their ability to act as efficient free radical scavengers, able to protect the cells against the oxidative damage caused by free radicals [28].

3.2. Repellent Activity. Blood-feeding insects are of great medical and veterinary importance, due both to their nuisance value and as vectors of disease [29]. Amongst insects that feed on humans, mosquitoes are of global importance [30]. In Amazonian regions, the use of plants as insect repellents is a common practice among Indio tribes. The majority of Itaputyr interviewed villagers admitted that they could not afford to use synthetic commercial mosquito repellents or insecticides because of high cost and/or discouraged by poor performance of some of the commercial products. Similar findings have been reported elsewhere in Africa, such as Guinea Bissau and Kenya, where the majority of the villagers could not afford synthetic commercial mosquito insecticides due to poverty [31, 32].

The results of mosquito protection in relation to application dose of different *B. orellana* dried seeds extracts are shown in Table 3. Skin repellent test showed the best results using a highly concentrated extract. Dried hexane extract resuspended in acetone provided the highest total percentage protection, followed by hydroalcoholic extract, with 90% and 73% of protection, respectively.

These results support the presence of mosquito repellent compounds in *B. orellana* seeds, particularly in the nonpolar fraction, also suggested by the Indio tradition to use the seeds to paint their bodies during open air religious and war ceremonies.

Table 3: Percentage of protection against mosquitoes (*A. aegypti*) in relation to the dose of the different *B. orellana* seed extracts (DW).

Extraction solvent	Concentration (mg/mL)	Protection (%)	SD (±)
Hexane	18.2	75%	16
	35.6	85%	18
	113.8	90%	18
EtOH	18.2	22%	13
	35.6	45%	14
	113.8	60%	16
$EtOH/H_2O$ (50 : 50 v/v)	18.2	23%	3
	35.6	50%	10
	113.8	73%	13

Data expressed as mean ± standard deviation ($n = 3$).

3.3. Volatile Organic Compounds (VOCs) Analysis. The volatile compounds extracted from headspaces of different *B. orellana* organs are presented in Table 4. At present, this represents the first report on the characterization of volatile compounds from different *B. orellana* organs by using the SPME technique. The SPME technique has the advantage of minimizing the sample handling and consequently decreasing the loss of volatile compounds. It is a simple and fast modern tool used to characterize the VOCs fingerprint of aromatic and medicinal plants and offers a valid alternative to hydrodistillation [33]. Moreover, this technique could represent a valid tool to analyze volatile compounds emitted by *in vivo* plants, sampling them *in situ,* in a nondisruptive way. Overall, 71 volatile compounds were identified in *B. orellana* samples with different proportions amongst the different plant organs.

The majority of volatile compounds present in the headspace from different *B. orellana* organs were sesquiterpenes, monoterpenes, hydrocarbons, and ketones. Simple terpenes have gained attention as agents of communication and defense against insects and can act as attractants or repellants.

Monoterpenes and sesquiterpenes are major components of many spices, fruit, and flower essential oils, having a great economic importance [34]. In the present study, we found a very scarce presence of monoterpenes especially α-pinene, that was absent in the *in vivo* samples. This is in contrast with Galindo-Cuspinera et al. that found α-pinene to be a major component isolated in the oil obtained from *B. orellana* samples. However, the volatile compounds were recovered using dynamic headspace-solvent desorption sampling and analyzed using GC-MS [35].

Sesquiterpenes constituted the major group of volatile compounds found in all the different *B. orellana* extracts analyzed. The major constituents were D-germacrene, δ-elemene, γ-elemene followed by β-caryophyllene, α-caryophyllene, γ-muurolene, and δ-cadinene.

Table 4: Identification of volatile organic compounds (VOCs) by HS-SME-GC/MS from headspace of *B. orellana* plant extracts obtained from different plant parts and from the headspace in living plant fruits (*in vivo* sampling).

RI[a]	Volatile compounds	Seed[b]		Wood[b]		Leaf[b]		Bark[b]		Fruit[b]		"*In vivo*"[c]	
		$n = 5$	s.d.	$n = 5$	s.d.	$n = 5$	s.d.	$n = 5$	s.d.	$n = 5$	s.d.	$n = 5$	s.d.
	Free fatty acids												
1285	Acetic acid	0.03	0.02	nd	—	0.01	—	nd	—	nd	—	nd	—
1628	2-ethylhexanoic acid	nd	—	nd	—	nd	—	nd	—	nd	—	0.12	0.01
	Total	**0.03**		—	—	**0.01**		—	—	—	—	**0.12**	
	Alcohols												
659	Ethanol	1.18	0.03	nd	—	nd	—	0.02		1.06	0.03	nd	—
848	2-methyl-1-propanol	0.01	—	nd	—	nd	—	nd	—	0.03	—	nd	—
919	2-pentanol	nd	—	nd	—	nd	—	nd	—	0.01	—	nd	—
976	1-penten-3-olo	nd	—	nd	—	nd	—	nd	—	0.01	—	nd	—
1040	2-methyl-1-butanol	0.13	0.05	nd	—	nd	—	nd	—	0.05	—	nd	—
1175	3-methyl-2-buten-1-ol	0.01		nd	—	nd	—	nd	—	nd	—	nd	—
1573	2-methoxyphenol	0.06		nd	—	nd	—	nd	—	nd	—	nd	—
1650	Phenol	0.01		nd	—	nd	—	nd	—	nd	—	nd	—
	Total	**1.39**		—	—	—	—	**0.02**		**1.16**		—	—
	Aldehydes												
645	2-methyl-butanal	nd	—	nd	—	0.01		nd	—	nd	—	nd	—
648	3-methyl-butanal	nd	—	nd	—	0.01		nd	—	nd	—	nd	—
687	Pentanal	nd	—	nd	—	0.03		nd	—	nd	—	nd	—
815	Hexanal	nd	—	nd	—	0.01		nd	—	nd	—	0.12	0.01
1034	2-hexenal	nd	—	nd	—	0.01		nd	—	nd	—	nd	—
1243	Nonanal	nd	—	nd	—	nd	—	nd	—	nd	—	0.17	0.02
1332	Decanal	nd	—	nd	—	nd	—	nd	—	nd	—	0.17	0.03
1341	Benzaldehyde	nd	—	2.20	0.04	nd	—	nd	—	nd	—	0.10	—
	Total	**—**	—	**2.20**		**0.07**		—	—	—	—	**0.56**	
	Ketones												
614	2-propanone	nd	—	nd	—	0.02		nd	—	nd	—	0.09	0.01
654	2-methyl-3-butanone	nd	—	0.71	0.01	nd	—	nd	—	nd	—	nd	—
685	3-pentanone	nd	—	2.50	0.02	nd	—	nd	—	0.07		nd	—
701	2-methyl-3-pentanone	nd	—	4.09	0.17	nd	—	nd	—	nd	—	nd	—
712	4-methyl-2-pentanone	nd	—	nd	—	nd	—	nd	—	nd	—	0.29	0.03
852	1,2-cycloctandione	0.01		nd	—	nd	—	nd	—	nd	—	nd	—
984	2-heptanone	nd	—	nd	—	nd	—	nd	—	0.01		nd	—
1121	3-hydroxy-2-butanone	nd	—	0.69	0.03	nd	—	nd	—	nd	—	nd	—
1189	6-methyl-5-hepten-2-one	0.02		nd	—	0.01		nd	—	nd	—	0.14	0.02
1422	4-tert-butylcyclohexanone	nd	—	nd	—	nd	—	nd	—	nd	—	0.50	0.06
	Total	**0.03**		**7.99**		**0.03**		—	—	**0.08**		**1.03**	
	Sulphur compounds												
604	Dimethyl sulfide	0.25	0.02	nd	—	nd	—	0.01	—	0.31	0.02	nd	—
	Total	**0.25**	—	—		—		**0.01**		**0.31**		—	—
	Furans												
639	2-methylfuran	0.02		2.71	0.15	0.01	—	0.01	—	nd	—	nd	—
671	2-ethylfuran	nd	—	nd	—	0.01	—	nd	—	nd	—	nd	—
	Total	**0.02**		**2.71**		**0.02**		**0.01**		—		—	

Table 4: Continued.

RI[a]	Volatile compounds	Seed[b]		Wood[b]		Leaf[b]		Bark[b]		Fruit[b]		"*In vivo*"[c]	
		$n = 5$	s.d.	$n = 5$	s.d.	$n = 5$	s.d.	$n = 5$	s.d.	$n = 5$	s.d.	$n = 5$	s.d.
	Esters												
618	Methyl acetate	0.01	—	nd	—	nd	—	nd	—	nd	—	nd	—
636	Ethyl acetate	0.51	0.12	nd	—	nd	—	nd	—	nd	—	nd	—
895	2-methyl-1-butanol acetate	0.03	—	nd	—	nd	—	nd	—	nd	—	nd	—
898	3-methyl-1-butanol ecetate	0.02	—	nd	—	nd	—	nd	—	nd	—	nd	—
	Total	**0.57**		—		—		—		—		—	
	Hydrocarbons												
746	Toluene	nd	—	nd	—	0.01		nd	—	nd	—	nd	—
884	Ethylbenzene	nd	—	2.24	0.06	nd	—	nd	—	nd	—	2.72	0.03
894	o-xylene	nd	—	nd	—	nd	—	nd	—	0.01		0.70	0.03
909	p-xylene	0.02		2.33	0.02	nd	—	0.01		nd	—	1.51	0.15
979	m-xylene	nd	—	0.73	0.09	nd	—	nd	—	0.01		0.50	0.05
1200	Dodecan	nd	—	nd	—	nd	—	nd	—	nd	—	0.67	0.03
1093	Styrene	0.01	0.01	6.15	0.12	0.01		0.03		nd	—	0.35	0.02
1166	2-ethenyl-1,1-dimethyl-3-methylenecyclohexane	nd	—	nd	—	0.52	0.03	nd	—	nd	—	nd	—
1270	Hydrocarbon	nd	—	nd	—	nd	—	nd	—	nd	—	1.11	0.08
1278	Methyl-(1-methylethenyl)-benzene	0.01	—	nd	—	nd	—	nd	—	0.12	0.08	nd	—
	Total	**0.04**		**11.45**		**0.54**		**0.04**		**0.14**		**7.56**	
	Mono-di-terpenes												
709	Triciclene	0.02		nd	—	nd	—	0.03	—	nd	—	nd	—
726	α-pinene	0.24	0.01	3.97	0.09	0.02	0.01	0.38	0.03	0.15	0.02	nd	—
735	α-thujene	nd	—	nd	—	nd	—	0.02	—	0.06	—	nd	—
776	Camphene	0.23	0.02	4.55	0.12	nd	—	0.98	0.05	0.10	—	nd	—
842	β-pinene	0.10	—	nd	—	nd	—	0.27	0.02	0.05	—	nd	—
876	Sabinene	0.02	—	nd	—	nd	—	0.09	0.01	0.03	—	nd	—
931	α-phellandrene	nd	—	nd	—	nd	—	0.01	—	0.01	—	nd	—
971	β-mircene	0.03	—	nd	—	0.19	0.03	0.13	0.01	0.41	0.02	0.11	0.01
978	α-terpinene	0.05	—	nd	—	0.01	—	nd	—	0.06	0.21	nd	—
1008	D-limonene	0.13		3.60	0.11	0.08	0.01	0.10	0.01	0.61	0.03	0.65	0.03
1081	Cis-ocimene	2.17	0.04	nd	—	3.19	0.05	3.30	0.08	19.99	0.37	3.38	0.08
1099	Trans-ocimene	0.05	0.01	nd	—	0.05	0.01	0.14	0.02	0.69	0.04	0.37	0.02
1111	Cymene	0.14	0.02	nd	—	0.03	0.01	nd	—	2.11	0.03	0.77	0.05
1127	α-terpinolene	0.05	0.01	nd	—	0.01	—	0.01	—	0.28	0.01	nd	—
1223	Allo ocimene isomer	nd	—	nd	—	0.01	—	0.02	—	0.73	0.03	nd	—
1227	Allo-ocimene	0.02		nd	—	nd	—	nd	—	0.10	0.01	0.10	0.02
1494	Naphthalene	nd	—	nd	—	nd	—	nd	—	nd	—	4.85	0.09
	Total	**3.25**		**12.12**		**3.59**		**5.48**		**25.39**		**10.23**	
	Sesquiterpenes												
1301	α-cubebene	0.39	0.13	2.43	0.24	1.91	0.07	1.19	0.06	nd	—	1.16	0.14
1316	δ-elemene	22.01	0.09	5.47	0.18	4.80	0.21	4.39	0.03	18.03	0.27	3.72	0.17
1321	α-ylangene	0.15	0.01	nd	—	0.64	0.02	8.15	0.02	0.57	0.03	nd	—
1328	α-copaene	2.53	0.07	7.39	0.04	11.44	0.15	4.20	0.03	3.25	0.05	2.80	0.08
1355	α-gurjunene	0.09	0.01	nd	—	0.39	0.01	0.99	0.02	nd	—	nd	—
1363	β-cubebene	0.65	0.05	nd	—	0.76	0.02	0.37	0.01	0.68	0.03	nd	—

Table 4: Continued.

RI[a]	Volatile compounds	Seed[b]		Wood[b]		Leaf[b]		Bark[b]		Fruit[b]		"*In vivo*"[c]	
		$n = 5$	s.d.	$n = 5$	s.d.	$n = 5$	s.d.	$n = 5$	s.d.	$n = 5$	s.d.	$n = 5$	s.d.
1387	Sesquiterpene	0.05	0.02	nd	—	0.10	0.02	0.09	0.02	nd	—	3.08	0.08
1390	Sesquiterpene	1.58	0.03	nd	—	1.00	0.07	0.35	0.06	nd	—	nd	—
1402	β-caryophyllene	1.80	0.03	11.02	0.54	22.39	0.18	6.68	0.14	nd	—	37.32	0.19
1410	Aromadendrene	0.16	0.01	2.91	0.08	3.21	0.03	11.60	0.08	nd	—	1.85	0.07
1413	Sesquiterpene	0.22	0.01	nd	—	nd	—	nd	—	7.73	0.24	nd	—
1424	Sesquiterpene	0.41		nd	—	1.07	0.02	1.40	0.03	0.96		nd	—
1435	Sesquiterpene	0.16		nd	—	nd	—	nd	—	nd	—	2.12	0.03
1440	Calarene	1.15	0.03	nd	—	0.45	0.01	0.76	0.01	2.19	0.02	nd	—
1452	α-caryophyllene	nd	—	8.70	0.25	12.00	0.07	7.12	0.17	nd	—	7.16	0.08
1454	Sesquiterpene	4.26	0.02	nd	—	0.55	0.01	nd	—	nd	—	nd	—
1462	Isoledene	1.80	0.04	nd	—	nd	—	0.43	0.01	3.41	0.19	nd	—
1469	Sesquiterpene	nd	—	nd	—	nd	—	nd	—	3.88	0.21	nd	—
1471	γ-muurolene	0.99	0.01	2.95	0.07	1.55	0.02	1.96	0.02	2.74	0.13	1.92	0.07
1476	β-cadinene	1.80	0.03	2.10	0.08	2.01	0.02	1.80	0.01	2.41	0.08	2.18	0.12
1486	D-germacrene	27.83	0.17	7.11	0.16	10.09	0.09	21.13	0.31	7.29	0.15	1.96	0.03
1487	β-selinene	nd	—	nd	—	0.83	0.02	0.66	0.02	nd	—	1.24	0.03
1496	α-selinene	nd	—	nd	—	0.51	0.03	0.53	0.01	1.81	0.08	nd	—
1499	γ-elemene	3.69	0.04	5.76	0.31	12.45	0.19	11.18	0.12	3.28	0.09	1.13	0.07
1516	Sesquiterpene	1.15	0.07	nd	—	nd	—	nd	—	nd	—	nd	—
1519	δ-cadinene	2.14	0.07	5.75	0.43	4.70	0.05	5.17	0.11	8.89	0.13	5.78	0.15
1527	Sesquiterpene	18.07	0.16	nd	—	1.47	0.03	2.73	0.09	0.23	0.01	nd	—
1531	1,2,3,4,4a,7-naphthalene hexahydro-1,6-dimethyl-4-(1-methylethyl)	0.10	—	nd	—	0.19	0.01	0.24	0.01	0.76	0.02	0.26	0.02
1537	α-muurolene	0.29	—	nd	—	0.22	0.01	0.24	0.02	1.54	0.09	0.46	0.02
1557	Sesquiterpene	0.82	0.02	nd	—	0.32	0.02	0.25	0.02	1.96	0.04	0.44	0.03
1563	Calamenene	nd	—	nd	—	0.05	—	0.07	0.01	0.19	0.01	0.99	0.02
1612	α-calacorene	nd	—	nd	—	0.02	—	0.03	0.01	0.08	0.01	0.64	0.01
1642	Sesquiterpene	nd	—	nd	—	nd	—	0.05	0.01	0.25	0.02	2.08	0.07
1663	Humulene oxide	nd	—	nd	—	0.05	—	0.03		nd	—	nd	—
1667	Nerolidol	nd	—	nd	—	0.15	0.01	nd	—	nd	—	nd	—
1691	Spathulenol	nd	—	nd	—	0.26	0.02	0.16		nd	—	0.84	0.02
1728	α-cadinol	0.01	—	nd	—	nd	—	nd	—	0.11	0.02	0.17	0.02
	Total	**94.3**		**61.59**		**95.58**		**93.95**		**72.24**		**79.3**	

[a]The Kovats retention index calculated for Rtx-Wax capillary column (25 m × 0.25 μm × 0.25 mm i.d.).
[b]Normalized amount of volatile compounds (percentage); (peak of volatile compound/total peak area of all volatile compounds) of *B. orellana* ($n = 3$) obtained from different parts.
[c]Normalized amount of volatile compounds (percentage); (peak of volatile compound/total peak area of all volatile compounds) of *B. orellana* living plants using *in vivo* sampling ($n = 3$).
s.d.: ± standard deviation.
nd: not detected.

Sesquiterpenes were significantly higher than mono-diterpenes in all plant organs analyzed. Leaves bark and seeds showed higher sesquiterpene concentration than wood and fruits dried or *in vivo* sampled. The total percentage of VOCs identified in the wood was rather low, only 61.59%, as compared to the other plant samples. Also, Zollo et al., found low levels of volatile compounds extracted from leaves and wood of *B. orellana*. The authors identified a great percentage of ishwarane, a sesquiterpene hydrocarbon not found in the present research [36].

Elemene and δ-cadinene have been found in all samples at different percentages. Minor sesquiterpenes found in *B. orellana* extracts that have distinctive aromas include cubebene, β-, and δ-cadinene. Cubebene has been described as having a fruity, sweet, citrus-like smell. β- and δ-cadinene are compounds occasionally used as fixatives in candy

flavours and have a dry-woody, slightly medicinal-tarry odour with some similarity to spices in the cumin-thyme family [37]. Some of the monoterpenes and sesquiterpenes found in our extracts have been previously described as having antimicrobial properties or as being associated with pharmacological properties [38]. D-germacrene, mainly found in dried seeds and leaves samples (27.83% and 21.13%, resp.), showed antimicrobial activity [39].

β-caryophyllene, mainly found in *in vivo* samples (37.32%), has been proved to have an anti-inflammatory effect by selectively binding cannabinoid receptors type 2 (CB_2) [37].

Cis-ocimene, mainly found in dried fruits samples (19.99%), showed antibacterial activity against Gram+ and Gram− (*Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa,* and *Klebsiella pneumoniae*) [40].

4. Conclusions

This study has renewed the state of knowledge about *B. orellana* bringing together the existing literature information and novelties such as VOCs profiling and indications about mosquito repellent capacity of seed extracts. At present, this research represents the first investigation on volatile compounds conducted on different *B. orellana* organs by means of a fast sample preparation method. In addition, SPME was confirmed to be a good technique to analyze volatile compounds emitted by *in vivo* plants, sampled *in situ,* in a nondisruptive way, with consequent potential big advantages for phytochemical and ecophysiological studies, particularly regarding rare and/or protected plants. General screening confirmed the literature data about phenol compounds content and radical scavenging capacity of *B. orellana* seed extracts.

The preliminary results on mosquito repellent activity confirmed the potential of the plant to protect against *A. aegypti* representing another theme of future investigation, fundamental in linking traditional value of a well-known plant with scientifically obtained proof of efficacy.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgment

The authors are grateful to the Itapury Cooperation Project for supporting the mission costs.

References

[1] C. Antweiler, "Local knowledge and local knowing: an anthropological analysis of contested "cultural products" in the context of development," *Anthropos*, vol. 93, no. 4–6, pp. 469–494, 1998.

[2] M. Coelho-Ferreira, "Medicinal knowledge and plant utilization in an Amazonian coastal community of Marudà, Parà State (Brazil)," *Journal of Ethnopharmacology*, vol. 126, no. 1, pp. 159–175, 2009.

[3] Brasil, *Política nacional de plantas medicinais e fitoterápicos, Ministério da Saúde, Secretaria de Ciência Tecnologia e Insumos Estratégicos*, Departamento de Assistência Farmacêutica, Brasília, Brazil, 2007.

[4] R. Rajendran, *Achiote:* Bixa Orellana *L. A Natural Food, Colour and Dye*, AES, College of Agricultural Science, Mangilao, Guam, 1st edition, 1990.

[5] P. R. N. Carvalho and M. Hein, "Urucum: uma fonte de corante natural," *Coletânea do ITAL*, vol. 19, pp. 25–33, 1989.

[6] A. T. Selvi, R. Aravindhan, B. Madhan et al., "Studies on the application of natural dye extract from *Bixa orellana* seeds for dyeing and finishing of leather," *Industrial Crops and Products*, vol. 43, pp. 84–86, 2013.

[7] M. G. Parra and L. M. Fidalgo, "Bixa orellana: properties and applications," in *Drug Plants IV*, V. K. Singh and J. N. Govil, Eds., pp. 25–35, 2010.

[8] M. P. Corrêa, *Dicionário das Plantas Úteis Do Brasil e das Exóticas Cultivadas*, vol. 4, Ministério da Agricultura/IBDF, Rio de Janeiro, Brazil, 1978.

[9] R. A. Voeks and A. Leony, "Forgetting the forest: assessing medicinal plant erosion in Eastern Brasil," *Economic Botany*, vol. 58, supplement 1, pp. S294–S306, 2004.

[10] M. T. L. A. Camargo, *Medicina Popular: Aspectos Metodologicos Para Pesquisa, Garrafada, Objeto de Pesquisa, Componentes Medicinais de Origem Vegetal, Animal e Mineral*, ALMED Editora e Livraria Ltda, Sao Paulo, Brasil, 1985.

[11] L. C. di Stasi, E. M. G. Santos, C. M. Dos-Santos, and C. A. Hiruma, *Plantas Medicinas da Amazonia*, UNESP Editora, Sao Paolo, Brasil, 1989.

[12] S. Patnaik, S. R. Mishra, G. B. Choudhury et al., "Phytochemical investigation and simultaneously study on anticonvulsant, antidiabetic activity of different leafy extracts of *Bixa orellana* Linn," *International Journal of Pharmaceutical and Biological Archives*, vol. 2, no. 5, pp. 1497–1501, 2011.

[13] K. Asokkumar, P. Jagannath, M. Umamaheswari et al., "Cardioprotective activity of *Bixa orellana* L. on isoproterenol induced myocardial necrosis in rats," *Journal of Pharmacy Research*, vol. 5, no. 4, pp. 1930–1934, 2012.

[14] A. T. Selvi, M. G. Dinesh, R. S. Satyanet et al., "Leaf and seed extracts of *Bixa orellana* L. exert anti-microbial activity against bacterial pathogens," *Journal of Applied Pharmaceutical Science*, vol. 1, no. 9, pp. 116–120, 2011.

[15] T. C. Fleischer, E. P. K. Ameade, M. L. K. Mensah, and I. K. Sawer, "Antimicrobial activity of the leaves and seeds of ,*Bixa orellana*," *Fitoterapia*, vol. 74, no. 1-2, pp. 136–138, 2003.

[16] A. G. Barrio, M. M. M. Grueiro, D. Montero et al., "*In vitro* antiparasitic activity of plant extracts from panama," *Pharmaceutical Biology*, vol. 42, no. 4-5, pp. 332–337, 2004.

[17] M. Viuda-Martos, G. L. Ciro-Gomez, Y. Ruiz-Navajas et al., "*In vitro* antioxidant and antibacterial activities of extracts from annatto (*Bixa orellana* L.) leaves and seeds," *Journal of Food Safety*, vol. 32, no. 4, pp. 399–406, 2012.

[18] B. Radhika, N. Begum, and K. Srisailam, "Pharmacognostic and preliminary phytochemical evaluation of the leaves of *Bixa orellana*," *Pharmacognosy Journal*, vol. 2, no. 7, pp. 132–136, 2010.

[19] M. L. T. Nguyen, "Cultivated plant collections from market places," *Ethnobotany Research & Applications*, vol. 3, pp. 5–15, 2005.

[20] U. P. Albuquerque, *Folhas Sagradas: as Plantas Liturgicas e Medicinais nos Cultos Afro-Bresileiros*, Nupeea, Recife, Brazil, 2nd edition, 2006.

[21] A. Giorgi, S. Panseri, M. S. Mattara, C. Andreis, and L. M. Chiesa, "Secondary metabolites and antioxidant capacities of *Waldheimia glabra* (Decne). Regel from Nepal," *Journal of the Science of Food and Agriculture*, vol. 93, no. 5, pp. 1026–1034, 2013.

[22] WHO, "Report of the WHO informal consultation on the evaluation and testing insecticides," Tech. Rep. CTD/WHO PES/IC/96.1, Control of Tropical Diseases Division, World Health Organization, Geneva, Switzerland, 1996.

[23] A. Giorgi, M. Madeo, G. Speranza, and M. Cocucci, "Influence of environmental factors on composition of phenolic antioxidants of *Achillea collina* Becker ex Rchb," *Natural Product Research*, vol. 24, no. 16, pp. 1546–1559, 2010.

[24] L. S. Anker, P. C. Jurs, and P. A. Edwards, "Quantitative structure-retention relationship studies of odor-active aliphatic compounds with oxygen-containing functional groups," *Analytical Chemistry*, vol. 62, no. 24, pp. 2676–2684, 1990.

[25] J. K. Moon and T. Shibamoto, "Antioxidant assays for plant and food components," *Journal of Agricultural and Food Chemistry*, vol. 57, no. 5, pp. 1655–1666, 2009.

[26] C. R. Cardarelli, M. D. T. Benassi, and A. Z. Mercadante, "Characterization of different annatto extracts based on antioxidant and colour properties," *LWT—Food Science and Technology*, vol. 41, no. 9, pp. 1689–1693, 2008.

[27] R. C. Chisté, A. Z. Mercadante, A. Gomes, E. Fernandes, J. L. F. D. C. Lima, and N. Bragagnolo, "*In vitro* scavenging capacity of annatto seed extracts against reactive oxygen and nitrogen species," *Food Chemistry*, vol. 127, no. 2, pp. 419–426, 2011.

[28] M. C. Castello, A. Phatak, N. Chandra, and M. Sharon, "Antimicrobial activity of crude extracts from plant parts and corresponding calli of *Bixa orellana* L.," *Indian Journal of Experimental Biology*, vol. 40, no. 12, pp. 1378–1381, 2002.

[29] A. Abdul Rahuman, "Efficacies of medicinal plant extracts against blood-sucking parasites," in *Nature Helps, Parasitology Research Monographs*, H. Mehlhorn, Ed., Springer, Berlin, Germany, 2011.

[30] J. Fang, "Ecology: a world without mosquitoes," *Nature*, vol. 466, pp. 432–434, 2010.

[31] A. Seyoum, K. Pålsson, S. Kung'a et al., "Traditional use of mosquito-repellent plants in western Kenya and their evaluation in semi-field experimental huts against *Anopheles gambiae*: ethnobotanical studies and application by thermal expulsion and direct burning," *Transactions of the Royal Society of Tropical Medicine and Hygiene*, vol. 96, no. 3, pp. 225–231, 2002.

[32] K. Karunamoorthi, K. Ilango, and A. Endale, "Ethnobotanical survey of knowledge and usage custom of traditional insect/mosquito repellent plants among the Ethiopian Oromo ethnic group," *Journal of Ethnopharmacology*, vol. 125, no. 2, pp. 224–229, 2009.

[33] A. Giorgi, S. Panseri, N. N. M. C. Nanayakkara, and L. M. Chiesa, "HS-SPME-GC/MS analysis of the volatile compounds of *Achillea collina*: evaluation of the emissions fingerprint induced by *Myzus persicae* infestation," *Journal of Plant Biology*, vol. 55, no. 3, pp. 251–260, 2012.

[34] S. Terashima, M. Shimizu, S. Horie, and N. Morita, "Studies on aldose reductase inhibitors from natural products. IV. Constituents and aldose reductase inhibitory effect of Chrysanthemum morifolium, *Bixa orellana* and *Ipomoea batatas*," *Chemical and Pharmaceutical Bulletin*, vol. 39, no. 12, pp. 3346–3347, 1991.

[35] V. Galindo-Cuspinera, M. B. Lubran, and S. A. Rankin, "Comparison of volatile compounds in water- and oil-soluble annatto (*Bixa orellana L.*) extracts," *Journal of Agricultural and Food Chemistry*, vol. 50, no. 7, pp. 2010–2015, 2002.

[36] P. H. A. Zollo, L. Biyiti, F. Tchoumbougnang, C. Menut, G. Lamaty, and P. Bouchet, "Aromatic plants of tropical Central Africa. Part II. Chemical composition and antifungal activity of thirteen essential oils from aromatic plants of Cameroon," *Flavour and Fragrance Journal*, vol. 13, pp. 107–114, 1998.

[37] J. Gertsch, M. Leonti, S. Raduner et al., "Beta-caryophyllene is a dietary cannabinoid," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 26, pp. 9099–9104, 2008.

[38] J. A. Shilpi, M. Taufiq-Ur-Rahman, S. J. Uddin, M. S. Alam, S. K. Sadhu, and V. Seidel, "Preliminary pharmacological screening of Bixa orellana L. leaves," *Journal of Ethnopharmacology*, vol. 108, no. 2, pp. 264–271, 2006.

[39] X. S. Wang, W. Yang, S. J. Tao et al., "The effect of δ-elemene on hela cell lines by apoptosis induction," *Yakugaku Zasshi*, vol. 126, no. 10, pp. 979–990, 2006.

[40] T. Jovanovic, D. Kitic, R. Palic, G. Stojanovic, and M. Ristic, "Chemical composition and antimicrobial activity of the essential oil of *Acinos arvensis* (Lam.) Dandy from Serbia," *Flavour and Fragrance Journal*, vol. 20, no. 3, pp. 288–290, 2005.

Hindawi Publishing Corporation
Journal of Chemistry
Volume 2013, Article ID 645086, 4 pages
http://dx.doi.org/10.1155/2013/645086

Research Article

Acetylcholinesterase Inhibitory Activities of Flavonoids from the Leaves of *Ginkgo biloba* against Brown Planthopper

Xiao Ding,[1] Ming-An Ouyang,[2] Xiang Liu,[3] and Rei-Zhen Wang[1]

[1] *Key Laboratory of Bio-Pesticide and Chemistry-Biology, Ministry of Education, Fujian Agriculture and Forestry University, Fuzhou, Fujian 350002, China*
[2] *Institute of Plant Virology, Fujian Agriculture and Forestry University, Fuzhou, Fujian 350002, China*
[3] *Key Laboratory of Integrated Pest Management for Fujian-Taiwan Crops, Ministry of Agriculture, Fuzhou, Fujain 350002, China*

Correspondence should be addressed to Ming-An Ouyang; maouyang@hqu.edu.cn

Received 13 October 2012; Revised 1 March 2013; Accepted 10 March 2013

Academic Editor: A. Hamid A. Hadi

Ginkgo biloba is a traditional Chinese medicinal plant which has potent insecticidal activity against brown planthopper. The MeOH extract was tested in the acetylcholinesterase (AChE) inhibitory assay with IC_{50} values of 252.1 μg/mL. Two ginkgolides and thirteen flavonoids were isolated from the leaves of *Ginkgo biloba*. Their structures were established on the basis of spectroscopic data interpretation. It revealed that the 13 isolated flavonoids were found to inhibit AChE with IC_{50} values ranging from 57.8 to 133.1 μg/mL in the inhibitory assay. AChE was inhibited dose dependently by all tested flavonoids, and compound **6** displayed the highest inhibitory effect against AChE with IC_{50} values of 57.8 μg/mL.

1. Introduction

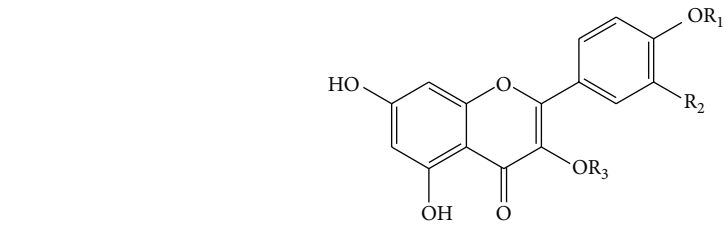
Ginkgo biloba (Ginkgoaceae) is the oldest living tree, with a long history of use in traditional Chinese medicine [1]. The extract of dried green leaves which was extracted with an acetone/water mixture has been standardized to contain 6% terpene trilactones (ginkgolides and bilobalide) and 24% flavonoids [2]. Those flavonoids are almost flavonol-*O*-glycosides, a combination of aglycones kaempferol, quercetin, and isorhamnetin, with glucose or rhamnose or both linking to different positions of the flavonol moiety [3]. Moreover, brown planthopper, *Nilaparvata lugens* (Homoptera: Delphacidae) is a major rice pest in many countries of Asia. And its infestation is initiated every year by a windborne from the tropics and subtropics. Acetylcholinesterase (AChE) is a substrate-specific enzyme that degrades the neurotransmitter acetylcholine in the nerve synapse [4]. As reported, linarin was isolated from *Buddleja davidii*, with a detection limit of 10 ng in the bioautographic TLC assay, which is at the same level as the known active compound galantamine [5].

In this study, we describe the isolation and structures as well as acetylcholinesterase inhibitory activity of thirteen flavonoids together with two ginkgolides from the leaves of *Ginkgo biloba* (Figures 1 and 2).

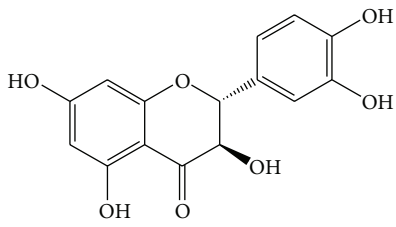
2. Experimental

2.1. Chemicals. Chlorpyrifos was purchased from Sigma Chemicals Co. (St. Louis, Mo, USA). Acetylcholine iodide (ACHI) was purchased from Fluka Chemical Co. (Milwaukee, WI, USA), and 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) was purchased from Biological Engineering Co. (Huzhou, Zhejiang, China). And other reagents used were of the highest available quality and obtained from China National Medicines Co., Ltd. (Beijing, China).

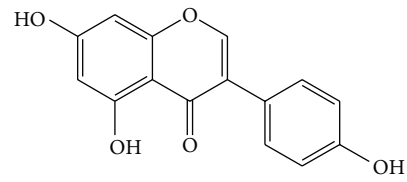
2.2. Plant Material. The plant materials, leaves of *Ginkgo biloba*, used in this study were purchased from Fujian Medicine Co., Ltd, Fujian Province, People's Republic of China, in April 2011, and identified by Professor Ke-Cuo He, College of Plant Protection, Fujian Agriculture and Forestry University. A voucher specimen (no. 110406) was deposited in the College of Plant Protection, Fujian Agriculture and Forestry University.



	R_1	R_2	R_3
1	H	H	H
2	H	OH	H
3	CH_3	H	CH_3
4	H	OH	Rha
5	H	OH	α-Glu
6	H	OH	β-Glu
7	H	OH	Rha-(1→2)-Glu-
8	H	OH	Rha-(1→6)-Glu-
9	CH_3	H	Rha-(1→6)-Glu-
10	H	OH	Rha-(1→4)-Rha-(1→6)-Glu
11	CH_3	H	Rha-(1→4)-Rha-(1→6)-Glu



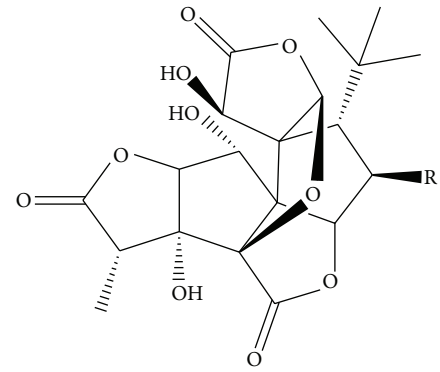
12



13

Figure 1: Structure of flavonoids.

2.3. Extraction and Isolation. The air-dried leaves of *Ginkgo biloba* (5.0 kg) were powdered and extracted exhaustively by maceration with MeOH at room temperature. The extract solution was concentrated under diminished pressure to afford a residue, which was partitioned between ethyl acetate (5 × 1 L) and water (5 × 1 L) to give 25 g and 34 g of extracts from these two layers, respectively. The ethyl acetate extract was subjected to column chromatography over silica gel and eluted with a gradient $CHCl_3$-MeOH (100 : 1-2 : 1) to afford eight fractions. Fr. **3** (3.2 g) was submitted to RP-18 column chromatography and eluted with 60–100% MeOH to afford three fractions. Afterwards, SFr. **2** (862 mg) was analyzed by normal phase semipreparative HPLC with $CHCl_3$-acetone (10 : 1) to yield **14** (139.8 mg) and **15** (221.0 mg). Fr. **5** (2.8 g) as mentioned above, was subjected to step-gradient silica gel column chromatography with a solvent consisting of $CHCl_3$-MeOH to afford five fractions. And then, SFr. **3** (365 mg) was subjected to column chromatography over RP-18 and eluted with a gradient of 40–90% MeOH. Further, it was purified by RP-18 semi-preparation HPLC and eluted with 85% MeOH to yield **1** (12.7 mg) and **3** (29.6 mg). The water extract was subjected to step-gradient silica gel column chromatography with a solvent consisting of $CHCl_3$-MeOH (20 : 1-1 : 1) to afford seven fractions. And Fr. **1** (303 mg) was purified over RP-18 with 80% MeOH to yield **2** (22.4 mg) and **6** (24.5 mg). Fr. **3** (303 mg) was purified over RP-18 with 75% MeOH to yield **4** (42.8 mg) and **5** (104.1 mg). Fr. **4** (2.6 g) was subjected to step-gradient silica gel column chromatography with a solvent consisting of $CHCl_3$-MeOH to afford **8** (23.1 mg), **11** (18.4 mg), and five fractions. SFr. **2** (356 mg) was purified by RP-18 semi-preparation HPLC and eluted with 85% MeOH to yield **9** (11.9 mg) and **12** (24.5 mg). Dealing with SFr. **4** (216 mg), it yielded **7** (16.9 mg) by RP-18 semi-preparation HPLC. SFr. **5** (216 mg) was purified by Sephadex LH-20 to yield **10** (15.3 mg) and **13** (17.8 mg) with MeOH.



	R
14	H
15	OH

Figure 2: Structure of ginkgolides.

2.4. Insect. The brown planthopper, used in this study was first collected from the experimental field of Fujian Agriculture and Forestry University, Fuzhou, Fujian China. Then, it was reared on rice seedling in laboratory, at 25 ± 1°C, 16 L/8 D.

2.5. Determination of IC_{50} of Test Compounds to AChE. One hundred 3rd instar larvae were homogenized in a glass homogenizer with 2 mL of 0.02 mol/L phosphate buffer (PH 7.0, containing 0.1% Triton X-100) using the method of Park and Choi [6], and the homogenizer was washed with 1 mL of phosphate buffer twice which was adapted for use in a microplate reader. The homogenate was centrifuged at 6000 r/min, 4°C for 30 min. the supernatant was used as the source of AChE. All compounds and chlorpyrifos were dissolved in acetone before diluting into the solution of AChE, and five concentrations of test compounds and chlorpyrifos solution were made by 2-fold dilution using acetone. 5 μL of test solution of each concentration was mixed with 95 μL of the solution of AChE, and the mixture was placed in wells of 96-well microtiter plate (Nunc, 300 μL volume per well) for 1 hour. Then, the 100 μL DTNB (0.3 mmol/L) and 100 μL ATCHI (1.5 mmol/L) were added successively. The residual activity of AChE was measured at 405 nm on the microplate reader [7]. A control reaction was carried out using phosphate buffer instead of test compounds and was considered as 100% activity. Inhibition, in %, was calculated in the following way:

$$I\,(\%) = 100 - \left(\frac{A_{\text{compound}}}{A_{\text{control}}}\right) \times 100, \quad (1)$$

where A_{compound} is the absorbance of the test compounds containing reaction and A_{control} is the absorbance of the reaction control. IC_{50} was obtained by plotting the inhibition percentage against using concentrations.

3. Results and Discussion

With the aim of researching active compounds by measuring acetylcholinesterase-inhibiting activity from the leaves of *Ginkgo biloba*, the MeOH extract and compounds **1–15** were tested in the acetylcholinesterase inhibitory assay. The MeOH extract proved to be active with IC_{50} = 252.1 μg/mL. In order to substantiate the result and find out which compound inhibited the enzyme, the extract was subjected to column chromatography over silica gel, RP-18, Sephadex LH-20, and semipreparation HPLC to yield the active compounds **1–13**.

*3.1. Structural Elucidation of Compounds **1–15**.* Thirteen flavonoids, namely, kaempferol (**1**) [8], quercetin (**2**) [9], ermanin (**3**) [10], quercetin-3-*O*-α-L-rhamnopyranoside (**4**) [11], quercetin-3-*O*-α-D-glucopyranoside (**5**), quercetin-3-*O*-β-D-glucopyranoside (**6**) [12], quercetin-3-*O*-α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranoside (**7**) [13], quercetin-3-*O*-α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranoside (**8**) [14], acacetin-3-*O*-α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranoside (**9**), quercetin-3-*O*-α-L-rhamnopyranosyl-(1 → 4)-*O*-α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranoside (**10**) [15], kaempferol-3-*O*-α-L-rhamnopyranosyl-(1 → 4)-*O*-α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranoside (**11**) [16], taxifolin (**12**), and genistein (**13**) [17]; two ginkgolides, namely, ginkgolide B (**14**) and ginkgolide C (**15**) [18] were obtained from the leaves of *Ginkgo biloba*. The structure determination of compounds **1–15** was established using NMR spectral method, and their spectral data were compared to previous literature values.

Table 1: AChE inhibitory activity of compounds **1–15**.

Compound	IC_{50} (μg/mL)[a]
1	100.5
2	95.8
3	88.5
4	110.9
5	79.4
6	57.8
7	77.5
8	73.1
9	97.8
10	112.6
11	101.7
12	133.1
13	103.8
14	—[b]
15	—
Chlorpyrifos	12.4

[a]All compounds were examined in a set of experiments repeated three times; IC_{50} values of compounds represent the concentration that caused 50% enzyme activity loss. [b]No inhibitory activity.

3.2. AChE Inhibitory Activity. The AChE inhibitory activity of the evaluated extract of *Ginkgo biloba* at different concentrations (31.25, 62.5, 125, 250, and 500 μg/mL) showed that the extract has moderate inhibitory activity with the IC_{50} = 252.1 μg/mL (Table 1). Ginkgolides B and C were found to be inactive in the AChE inhibitory test, while their effect target seemed to be GABA receptor [19].

Flavonoids have previously been reported to be multipotent agents in combating Alzheimer's disease (AD) by enhancing acetylcholine levels. Among the 13 isolated flavonoids, they were found to inhibit AChE with IC_{50} values ranging from 57.8 to 133.1 μg/mL in the inhibitory assay. AChE was inhibited dose dependently by all tested flavonoids. As shown in Table 1, compound **6** exerted the most promising activity with IC_{50} values of 57.8 μg/mL following by the inhibitory property of compound **8** that exhibited an IC_{50} value of 73.1 μg/mL as compared with a positive control, chlorpyrifos (IC_{50} = 12.4 μg/mL). Compounds **4, 10**, and **12** showed weak inhibitory activity, with IC_{50} values of 110.9, 112.6, and 133.1 μg/mL, respectively.

4. Conclusion

In conclusion, we isolated two ginkgolides and thirteen flavonoids from the leaves of *Ginkgo biloba*. The AChE inhibitory activity of test compounds revealed that flavonoids were found to inhibit AChE with IC_{50} values ranging from 57.8 to 133.1 μg/mL, and that ginkgolides were inactive. These

inhibitors enhance the signal transmission in nerve synapses by prolonging the effect of acetylcholine, which is harmful to brown planthopper. The isolation and identification of AChE inhibitors of these compounds may be of interest to clarify the physiological role of this enzyme and to provide novel pesticide.

Conflict of Interests

There is no conflict of interests among all authors.

Acknowledgments

This research was financially supported by Fujian Natural Science Foundation (Grant no. 2012J01088), Chinese Ministry of Education (Grant no. 201251511007), and the Ministry of Science and Technology Foundation (Grant no. 2011AA10A203).

References

[1] R. T. Major, "The Ginkgo, the most ancient living tree," *Science*, vol. 157, no. 3794, pp. 1270–1273, 1967.

[2] K. Drieu and H. Jaggy, "History, development and constituents of EGb 761," *Medicinal and Aromatic Plants-Industrial Profiles*, vol. 12, pp. 267–277, 2000.

[3] K. Strømgaard and K. Nakanishi, "Chemistry and biology of terpene trilactones from *Ginkgo biloba*," *Angewandte Chemie*, vol. 43, no. 13, pp. 1640–1658, 2004.

[4] A. Borloz, A. Marston, and K. Hostettmann, "The determination of huperzine A in European Lycopodiaceae species by HPLC-UV-MS," *Phytochemical Analysis*, vol. 17, no. 5, pp. 332–336, 2006.

[5] A. Marston, J. Kissling, and K. Hostettmann, "A rapid TLC bioautographic method for the detection of acetylcholinesterase and butyrylcholinesterase inhibitors in plants," *Phytochemical Analysis*, vol. 13, no. 1, pp. 51–54, 2002.

[6] H. M. Park and S. Y. Choi, "Changes in esterase activity and acetylcholinesterase sensitivity of insecticide-selected strains of the brown planthopper (*Nilaparvata lugens* Stal)," *Korean Journal Plant Protection*, vol. 30, no. 2, pp. 117–123, 1991.

[7] Z. W. Liu and Z. J. Han, "The roles of carboxylesterase and AChE insensitivity in malathion resistance development in brown planthopper," *Acta Entomologica Sinica*, vol. 46, no. 2, pp. 250–253, 2003.

[8] S. P. Jun, S. R. Ho, H. K. Duck, and S. C. Ih, "Enzymatic preparation of kaempferol from green tea seed and its antioxidant activity," *Journal of Agricultural and Food Chemistry*, vol. 54, no. 8, pp. 2951–2956, 2006.

[9] X. Q. Wang, C. J. Zhou, N. Zhang, G. Wu, and M. H. Li, "Studies on the chemical constituents of *Artemisia lavandulaefolia*," *China Journal of Chinese Materia Medica*, vol. 34, no. 2, pp. 234–236, 2011.

[10] J. Martinez, A. M. Silván, M. J. Abad, P. Bermejo, A. Villar, and M. Söllhuber, "Isolation of two flavonoids from *Tanacetum microphyllum* as PMA-induced ear edema inhibitors," *Journal of Natural Products*, vol. 60, no. 2, pp. 142–144, 1997.

[11] R. A. Mothana, M. S. Al-Said, A. J. Al-Rehaily et al., "Anti-inflammatory, antinociceptive, antipyretic and antioxidant activities and phenolic constituents from *Loranthus regularis* Steud. ex Sprague," *Food Chemistry*, vol. 130, no. 2, pp. 344–349, 2012.

[12] K. Ioku, T. Tsushida, Y. Takei, N. Nakatani, and J. Terao, "Antioxidative activity of quercetin and quercetin monoglucosides in solution and phospholipid bilayers," *Biochimica et Biophysica Acta*, vol. 1234, no. 1, pp. 99–104, 1995.

[13] A. Sinz, R. Matusch, T. Santisuk, S. Chaichana, and V. Reutrakul, "Flavonol glycosides from *Dasymaschalon sootepense*," *Phytochemistry*, vol. 47, no. 7, pp. 1393–1396, 1998.

[14] M. Haribal and J. A. A. Renwick, "Oviposition stimulants for the monarch butterfly: flavonol glycosides from *Asclepias curassavica*," *Phytochemistry*, vol. 41, no. 1, pp. 139–144, 1996.

[15] T. Sekine, J. Arita, A. Yamaguchi et al., "Two flavonol glycosides from seeds of *Camellia sinensis*," *Phytochemistry*, vol. 30, no. 3, pp. 991–995, 1991.

[16] M. A. Beck and H. Häberlein, "Flavonol glycosides from *Eschscholtzia californica*," *Phytochemistry*, vol. 50, no. 2, pp. 329–332, 1999.

[17] Y. Shi, R. B. Shi, B. Liu, Y. R. Lu, and L. J. Du, "Isolation and elucidation of chemical constituents with antiviral action from Yinqiaosan on influenza virus," *Zhongguo Zhongyao Zazhi*, vol. 28, no. 1, pp. 46–47, 2003.

[18] M. C. Woods, I. Miura, Y. Nakadaira, A. Terahara, M. Maruyama, and K. Nakanishi, "The ginkgolides. V. Some aspects of their NMR spectra," *Tetrahedron Letters*, vol. 8, no. 4, pp. 321–326, 1967.

[19] S. H. Huang, R. K. Duke, M. Chebib, K. Sasaki, K. Wada, and G. A. R. Johnston, "Ginkgolides, diterpene trilactones of *Ginkgo biloba*, as antagonists at recombinant $\alpha 1\beta 2\gamma 2$L GABA A receptors," *European Journal of Pharmacology*, vol. 494, no. 2-3, pp. 131–138, 2004.

Hindawi Publishing Corporation
Journal of Chemistry
Volume 2013, Article ID 635079, 4 pages
http://dx.doi.org/10.1155/2013/635079

Research Article

Synthesis and Biological Activity of 2,5-Bisubstituted Derivatives of 1,3,4-Thiadiazol-2,5-dithiol

T. S. Zhivotova, R. E. Bakirova, S. D. Fazylov, S. K. Kabieva, and T. V. Kryazheva

Institute of Organic Synthesis and Carbochemistry of the Republic of Kazakhstan, Karaganda, Karaganda State Medical University, Karaganda State Technical University, Karaganda, Kazakhstan

Correspondence should be addressed to S. D. Fazylov; iosu8990@mail.ru

Received 18 July 2012; Revised 10 January 2013; Accepted 5 February 2013

Academic Editor: Mehmet Emin Duru

By reaction of 1,3,4-thiadiazol-2,5-dithiol with different organohalogens, chlorides of carboxylic acids, acrylic acid derivatives, alkaloids, and secondary amines, various derivatives of 2,5-bi-substituted 1,3,4-thiadiazole were synthesized, and biological properties of some of them were studied.

Substances containing a fragment of the structure of 1,3,4-thiadiazole have different physiological activity [1–4]. However, it is worth noting the small amount of 1,3,4-thiadiazol-2,5-dithiol, in spite of the availability and simplicity of its synthesis [5]. 1,3,4-Thiadiazol-2,5-dithiol due to the presence in its structure along with thiadiazol cycle, which is characterized by the presence of three donor centers—two atoms of nitrogen and sulfur heterocyclic atom—two equivalent sterically accessible thiol groups, has a high nucleophilicity and reactive ability and is an interesting object for chemical modification, which allows to introduce other functional groups and pharmacophore and synthesis of various 2,5-bisproizvodnye 1,3,4-thiadiazoles. In addition, data on the combination in one molecule thiadiazol cycle and alkaloid fragments are not accessible.

In order to obtain new biologically active substances with 1,3,4-thiadiazol fragment processes of alkylation and acylation, electrophilic and nucleophilic addition of 1,3,4-thiadiazol-2,5-dithiol to the α,β-unsaturated compounds were studied, the oxidation of thiol groups of 1,3,4-thiadiazol-2,5-dithiol into 1,3,4-thiadiazol-2,5-disulfonic acid was carried out. In addition, chelate salts of 1,3,4-thiadiazol-2,5-disulfonic acid and the initial 1,3,4-thiadiazol-2,5-dithiol with alkaloids and cyclic secondary amines were obtained. The combination of fragments of 1,3,4-thiadiazole, alkaloids and their structural analogues—piperidine and morpholine—according to our assumptions, should lead to a wide range of biologically active substances.

The alkylation reaction of 1,3,4-thiadiazol-2,5-dithiol by ¡?bhlt?¿organohalogens was carried out in two ways: in an alcohol medium in the presence of triethylamine as an acceptor of hydrogen halogens in the presence of sodium ethoxide. Alkylation takes place only on the thiol groups through S_N2 mechanism with the formation of biproducts (1–4). Yield of final products (1–4) during the alkylation in the presence of triethylamine constitutes 35–55% and, in the presence of sodium ethoxide, 58–80%. The increase in output (1–4) by about 25% indicates that the intermediate sodium salt is stronger electrolyte compared to the ammonium salt or initial 1,3,4-thiadiazol-2,5-dithiols.

Acylation of 1,3,4-thiadiazol-2,5-dithiol by chlorides of carboxylic acids was carried out in the presence of triethylamine with a slight warming of the reaction mixture for 6–20 hours with the formation of bis(acyl) derivatives (5–9). Outputs of final products (5–9) depended on the nature of the acylating reagent and ranged from 43 to 97%. The lowest yield of acylated product (9) is obtained by using butyric acid chloride, which is explained by electron donor influence of alkyl radical on the magnitude of the positive charge on the carbonyl atom of carbon in the initial chloride. Outputs of acylated products (5–8) are sufficiently high, but

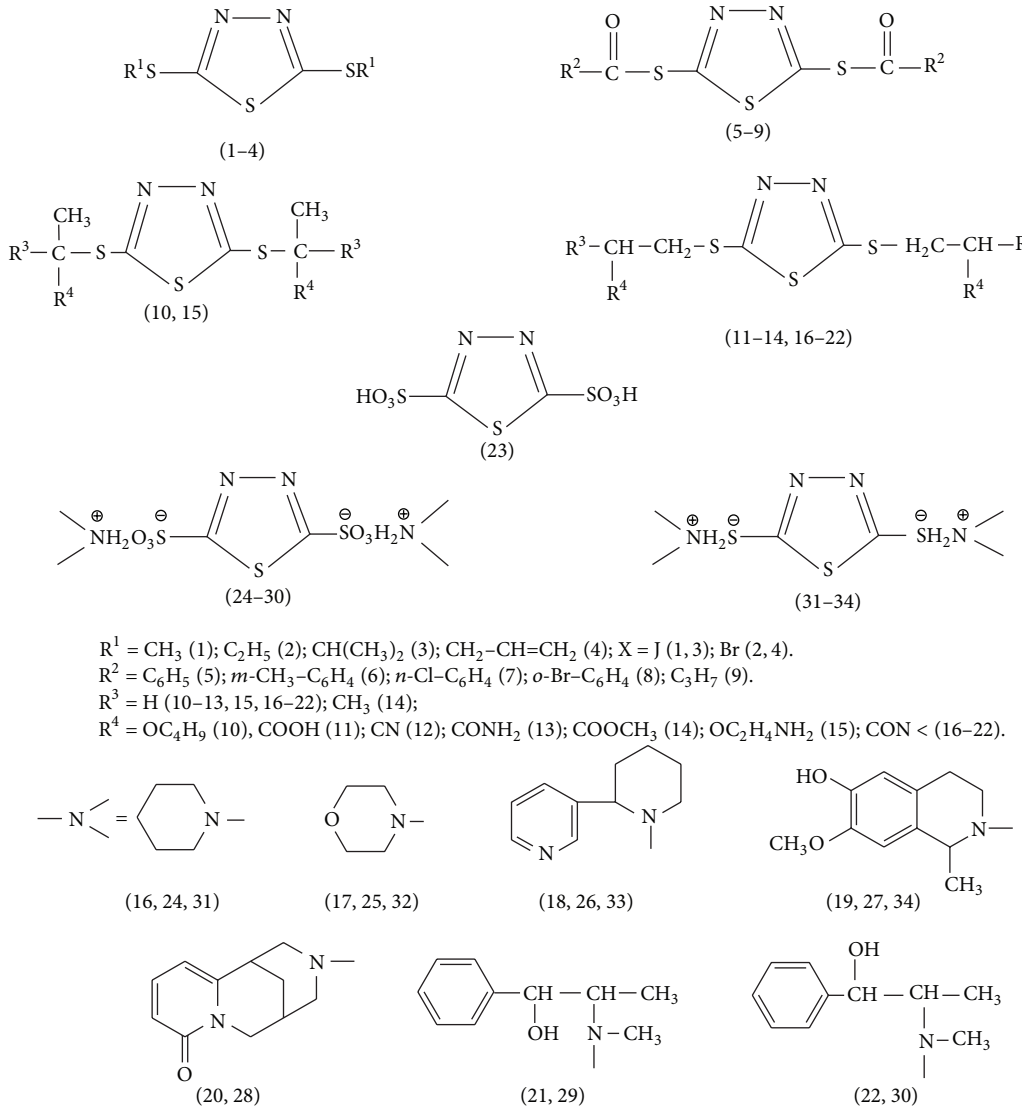


Figure 1

they depend on the nature of the substituent and its position in the benzene ring of the chloride (see Figure 1).

Depending on the nature of the substituent in the α-position of double bond the joining of 1,3,4-thiadiazol-2,5-dithiol to compounds containing a double bond may take place both by Markovnikov's rule and against it.

As the compounds containing the vinyl fragment acrylic acid, acrylamide, acrylonitrile, methyl methacrylate, vinyl ether of monoethanolamine, vinylbutyl ether, and N-substituted acrylamides were used, synthesis of compounds (10–22) was carried out in an alcoholic or aqueous-alcoholic medium with moderate heating and vigorous stirring of the reaction medium for 6–12 hours.

The presence of electron-withdrawing substituents in the α-position of the double bond (acrylic acid, acrylonitrile, acrylamide, acrylic acid methyl ester, and N-substituted acrylamides) leads to the fact that the electrophilic attack center of dithiol anion is β-carbon atom in double bond. The formation of carbanion and subsequent addition of a proton on the α-carbon atom occurs; that is, accession takes place against the Markovnikov rule with the formation of compounds (11–14, 16–22).

The presence of electron-donating substituents in vinylbutyl ether and vinyl ether of monoethanolamine significantly reduces the positive charge on the β-carbon atom of the double bond and leads to the fact that hydrogen atom attacks it, and a dithiol anion attaches to the α-carbon atom; that is, 1,3,4-thiadiazol-2,5-dithiol serves as the electrophile and joins to vinyl ethers with Markovnikov's rule with the formation of compounds (10, 15). Yields of compounds (10, 15) are 27 and 79%, respectively. The reactivity of monoethanolamine vinyl ether is significantly reduced due to the presence of intramolecular hydrogen bond in its molecule. The reaction rate and yields of compounds (11–14)

Table 1: Antioxidant activity of compounds (23–26, 28, and 29).

Compound number	Compound	C_{MDA}, nmol/L (30 min incubation)	Decrease of C_{MDA} in comparison to control level, %
Control	—	30,01	—
Ionol	2,6-Di-*tert*-butylphenol	9,32	68,94
23	1,3,4-Thiadiazol-2,5-disulfonic acid	15,16	50,52
24	Piperidine salt	4,58	84,74
25	Morpholine salt	14,68	51,08
26	Anabasine salt	20,68	31,09
28	Cytisine salt	14,68	51,08
29	*l*-ephedrine salt	14,05	53,18

(42–93%) depend on the electronic and steric factors that determine the activity and the availability of β-carbon atom in an acrylic system.

Despite the voluminous amide fragment in the alkaloids of N-alkaloid(amino)-substituted acrylamides, compared with acrylamide yields of final products (16–22) were 25–65%. Outputs (16–22) depend on the electron properties of the alkaloid (amino) amide fragment in the balance and on the conformational rigidity of the initial cycles of secondary amines and alkaloids, which does not allow to escape β-carbon atom of the double bond. The highest yield of obtained compounds (16, 17, 20) is due to the greater basicity and conformational rigidity of the initial cycles of piperidine, morpholine, and cytisine compared with the conformationally unstable anabasine, *l*-ephedrine, and *d*-pseudoephedrine. The low yield of compound (19) can be explained by spatial inaccessibility of the reaction center due to close proximity to fragment of salsoline undergoing nucleophilic attack by a double bond.

Oxidation of 1,3,4-thiadiazol-2,5-dithiol with an aqueous solution of potassium permanganate takes place only at the thiol groups without affecting the heterocyclic sulfur atom and leads to the formation of 1,3,4-thiadiazol-2,5-disulfonic acid (23) with the yield of about 98%. The reaction of the latter with the alkaloids and secondary amines complex salts (24–30) was synthesized with 50–77% yield. Similarly, the salt of 1,3,4-thiadiazol-2,5-dithiol (31–34) was obtained with yield of 78–94%. Synthesis was carried out in alcoholic medium by heating and stirring the reaction mixture for 5-6 hours. The outputs of salts (24–30) and (31–34) are in the same dependency on basicity and conformational rigidity of the cycles of the secondary amines and alkaloids (16–22).

The composition and structure of all synthesized compounds (1–34) were proved by IR, NMR ^{1}H, and ^{13}C spectroscopy.

In IR spectrums of all the synthesized compounds (1–34), there are absorption bands at 780–730 (C–S$_r$), 1060–1040, 1160–1120, 1270–1250 (S–C–S, N=C–S, N–N), 1460, and 1390 (N=C) cm^{-1}, which are assigned to thiadiazol cycle. In spectrums of ^{13}C NMR of compounds (1–34) signals of thiadiazol carbon cycle appear as a singlet in the range 168.0–140.0 ppm depending on the nature of the substituent at the sulfur atom of the thiol groups. Spectrums of ^{1}H NMR confirm the presence of proton signals in the substituents of compounds (1–34) in their characteristic regions of the spectrums.

Antibacterial and insecticidal activity of the original 1,3,4-thiadiazol-2,5-dithiol was detected. Studies on the antibacterial activity were carried out by conventional methods used for antibiotics [6]. We determined the sensitivity of microorganisms to a medicine by using method of serial dilutions (8.0, 4.0, 2.0, 1.0, 0.5, 0.25, and 0.125 mg/mL) in liquid medium. For these studies we have used cultures of microorganisms: *S. aureus 505, P. vulgaris 1, P. aeruginosa ATC 464, E. coli M-17, B. subtilis ACCC 6633*, as well as clinical strains of *S. agalactiae* and *C. albicans*.

The minimum bactericidal concentration (MBC) was determined by reseeding from the liquid medium, where there was no visible growth, on solid nutrient medium. The minimum bacteriostatic concentration (MSC) was evaluated by using turbidimetric method by comparing the intensity of microbial growth in liquid nutrient media. The results were recorded on a spectrophotometer, a control culture medium with the appropriate concentration of the drug experience was used.

It was determined that 1,3,4-thiadiazol-2,5-dithiol has antibacterial (MBC from 0.25 to 0.5 mg/mL; MSC from 0.125 to 0.25 mg/mL) (on all subjected strains of microorganisms) and antifungal (MBC 0.25 mg/mL, MSC 0.125 mg/mL) (on a clinical strain of *C. albicans*) activity.

Studies of pesticide (insecticide and aphicide) activity of 1,3,4-thiadiazol-2,5-dithiol were conducted in accordance with the methodological instructions [7]. Insecticidal activity of 1,3,4-thiadiazol-2,5-dithiol was studied in relation to the apple moth (*Hyponomeuta malinellus* Z.), while aphicide one to the apple aphid (Aphis pomi De Geez) and compared with those of standard preparations of Sumi-Alpha and Carbophos. 0,2% aqueous solution of 1,3,4-thiadiazol-2,5-dithiol was used for spraying. Aqueous solutions of reference substances were prepared according to the instructions for use. Average damage of plants before and after processing was registred and effectiveness of drugs calculated. The data obtained were processed statistically; the criterion of reliability was calculated by nonparametric methods [8].

As a result of the tests it was determined that 1,3,4-thiadiazol-2,5-dithiol has insecticidal activity against the

apple moth, exceeding the level of reference preparations of Sumi-Alpha and Carbophos. The mean value of efficiency for 1,3,4-thiadiazol-2,5-dithiol is 43,3%, for sumi-alpha: 38,0%, and carbophos: 19,3%. 1,3,4-thiadiazol-2,5-dithiol has also shown aphicidic activity against apple aphid, but the effectiveness of it is inferior to reference drugs.

According to the results of the primary biotrials 1,3,4-thiadiazol-2,5-dithiol can be recommended for in-depth studies to explore the possibility of its application in agriculture as a pesticide.

Antioxidant activity of 1,3,4-thiadiazol-2,5-disulfonic acid (23) and its salts (24–26, 28, 29) were tested to determine the effect of the thiadiazol ring and sulfonic groups in the structure of nitrogen-containing heterocyclic compounds on the antioxidant activity (AOA) of the latter.

Despite the fact that antioxidants are traditionally considered to be a substance of phenolic nature [9], nitrogen heterocycles containing piperidine, pyridine, pyrimidine, thiazole and other fragments found to have anti- or prooxidant action [10]. Antioxidant activity of 1,3,4-thiadiazol-2,5-disulfonic acid (23) and its salts (24–26, 28, 29) were studied using a model of liposomic oxidation of phosphatidylcholine with the test of thiobarbituric acid [11]. The inhibition of oxidation processes was assessed by measuring concentrations of malondialdehyde (MDA) after 10, 15, 20, and 30 minutes after the start of the reaction. Table 1 shows the antioxidant activity of compounds (23–26, 28, 29) compared to the comparison drug—a synthetic antioxidant ionol 30 minutes after the start of the reaction.

Thus, all studied compounds in varying degrees have shown antioxidant properties. Given the fact that anabasine and *l*-ephedrine by themselves do not exhibit antioxidant properties and cytisine is prooxidant [10], we can assume that it is the introduction of sulfonic acid groups, and thiadiazol fragment in the structure of alkaloids and secondary amines leads to occurrence of antioxidant abilities.

References

[1] L. Labanauskas, B. Kaltsas, E. Udrenayte, V. Buchinskayte, A. Brukshtus, and I. Susvilo, "Search for anti-inflammatory drugs among the derivatives of 1,2,4-triazole-5-thiol and 2-amino-1,3,4-thiadiazole," in *Proceedings of International Conference on Chemistry and Biological Activity of Nitrogen Heterocycles and Alkaloids*, p. 181, 2001.

[2] T. M. Salimov, M. A. Kukaniev, I. T. Sattorov, and D. M. Osimov, "Synthesis and antimicrobial activity of 2-bromo-7-methyl-5-oxo-5H-1,3,4- thiadiazolo[3,2-a]pyrimidine," *Pharmaceutical Chemistry Journal*, vol. 39, no. 6, pp. 311–312, 2005.

[3] W. Houbin, J. Shukui, M. Shufen, and Q. Zhaohai, "The study of pyridine derivatives. VIII. Synthesis and herbicidal activity of 2-(2-chloro-4-pyridyl)-5-alkylamino-1, 3, 4-thiadiazol," *Journal of China Agricultural University*, vol. 9, no. 1, pp. 63–66, 2004.

[4] D. Vullo, M. Franchi, E. Gallori, J. Antel, A. Scozzafava, and C. T. Supuran, "Carbonik anhydrase inhibitors Inhibition of mitochondrial isozyme V with aromatic and heteroziclic sulfonamides," *Journal of Medicinal Chemistry*, vol. 47, no. 5, pp. 1272–1279, 2004.

[5] B. A. Arbuzov and E. N. Uhvatova, "The synthesis of some esters of phosphinic and phosphoric acid," *Journal of Organic Chemistry*, vol. 29, no. 2, pp. 503–506, 1959.

[6] S. M. Navashin and I. P. Fomina, *Rational Antibiotic Therapy (Reference), Medicina*, Meditsina, Moscow, Russia, 1983.

[7] I. G. Berim, "Chemical protection of plants," S.-Pb.: Science, 1996.

[8] G. F. Lakin, *Biometrics*, Chemistry, Moscow, Russia, 1990.

[9] A. S. Seytembetova and S. M. Adekenov, *Natural Phenolic Compounds—A Promising Source of Antioxidants*, KazgosINTI, Almaty, Kazakhstan, 2001.

[10] T. S. Seytembetov, S. M. Adekenov, and E. D. Dalenov, *Antioxidants and Initiated Chemiluminescence*, University of KSMA, Akmola, Kazakhstan, 1996.

[11] L. D. Smirnov and K. M. Dumaev, "Oxiderivatives six-membered nitrogen heterocycles. Synthesis, inhibitory activity and biological properties," *Chemical Pharmaceutical Journal*, no. 4, pp. 38–44, 1983.

Hindawi Publishing Corporation
Journal of Chemistry
Volume 2013, Article ID 717232, 6 pages
http://dx.doi.org/10.1155/2013/717232

Research Article

Acetylcholinesterase and Butyrylcholinesterase Inhibitory Activities of β-Carboline and Quinoline Alkaloids Derivatives from the Plants of Genus *Peganum*

Ting Zhao,[1] Ke-min Ding,[1] Lei Zhang,[1] Xue-mei Cheng,[1,2,3] Chang-hong Wang,[1,2,3] and Zheng-tao Wang[1,2,3]

[1] *Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine, 1200 Cailun Road, Zhangjiang Hi-Tech Park, Shanghai 201210, China*
[2] *The MOE Key Laboratory for Standardization of Chinese Medicines and The SATCM Key Laboratory for New Resources and Quality Evaluation of Chinese Medicines, Shanghai 201210, China*
[3] *Shanghai R&D Center for Standardization of Chinese Medicines, Shanghai 201210, China*

Correspondence should be addressed to
Chang-hong Wang; wchcxm@hotmail.com and Zheng-tao Wang; wangzht@hotmail.com

Received 29 June 2012; Accepted 29 January 2013

Academic Editor: A. Hamid A. Hadi

It was reported that the main chemical constituents in plants of genus Peganum were a serial of β-carboline and quinoline alkaloids. These alkaloids were quantitatively assessed for selective inhibitory activities on acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) by *in vitro* Ellman method. The results indicated that harmane was the most potent selective AChE inhibitor with an IC_{50} of 7.11 ± 2.00 μM and AChE selectivity index (SI, IC_{50} of BChE/IC_{50} of AChE) of 10.82. Vasicine was the most potent BChE inhibitor with feature of dual AChE/BChE inhibitory activity, with an IC_{50} versus AChE/BChE of 13.68 ± 1.25/2.60 ± 1.47 μM and AChE SI of 0.19. By analyzing and comparing the IC_{50} and SI of those chemicals, it was indicated that the β-carboline alkaloids displayed more potent AChE inhibition but less BChE inhibition than quinoline alkaloids. The substituent at the C7 position of the β-carboline alkaloids and C3 and C9 positions of quinoline alkaloids played a critical role in AChE or BChE inhibition. The potent inhibition suggested that those alkaloids may be used as candidates for treatment of Alzheimer's disease. The analysis of the quantitative structure-activity relationship of those compounds investigated might provide guidance for the design and synthesis of AChE and BChE inhibitors.

1. Introduction

Alzheimer's disease (AD) was characterized by dementia that typically begins with subtle recognition failure and poor memory. It slowly becomes more severe and, eventually, incapacitating. The cholinergic system seemed particularly susceptible to synapse loss, especially in cortical regions associated with memory and executive function [1]. Recent studies showed that the main cause of the loss of cognitive functions in AD patients was a continuous decline of the cholinergic neurotransmission in cortical and other regions of the human brain [2]. Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are hydrolytic enzymes that act on acetylcholine (ACh) to terminate its actions in the synaptic cleft by cleaving the neurotransmitter to choline and acetate. Both enzymes are present in the brain and detected in neurofibrillary tangles and neuritic plaques. It was suggested that AChE predominates in the healthy brain, with BChE considered to play a minor role in regulating brain ACh levels. However, BChE activity progressively increases in patients with AD, while AChE activity remains unchanged or declines. Both enzymes therefore represent legitimate therapeutic targets for ameliorating the cholinergic deficit considered to be responsible for the declines in cognitive, behavioral, and global functioning characteristics of AD [3]. Despite the unknown etiology of AD, those findings supported that it was needed to control the activity of the cholinesterase (ChE) at different stages of AD progression. One of the most effective

treatment strategies was suggested to restrain cholinergic function and elevate ACh level through inhibiting AChE and BChE. Therefore, AChE and BChE inhibitors were developed for the treatment of AD [4, 5].

The history of drug discovery showed that plants were rich sources of new active compounds, and many synthetic drugs owed their origin to plant-based medicine. The genus *Peganum* consists of six species and one subspecies and three of them, that is, *P. harmala* Linn, *P. nigellastrum* Bunge, and *P. multisectum* (Maxim.) Bobr, are mainly distributed in the arid and semiarid areas in the northwest of China [6]. *P. harmala* is a well-known and effective herbal medicine in Turkey, Iran, and China [6–8]. The seeds and whole plants appear to possess medicinal properties and can be used to treat various ailments including rheumatism, hypertension, diabetes, and asthma jaundice and as antiparasitic agents [6, 9–11]. It was reported that the main chemical constituents in the seeds and whole plant of genus *Peganum* were a serial of β-carboline and quinoline alkaloids [12, 13].

As part of the same project, we first reported the screening of AChE inhibition activity of seeds extracts from genus *Peganum* with rapid bioautographic assay on TLC plates [14, 15]. It was found that the alkaloids fraction (mainly β-carboline and quinoline alkaloids) showed potential inhibitory effects on the AChE activity. A number of alkaloids including two new compounds nigellastrine I and nigellastrine II, along with eight known alkaloids, vasicinone, vasicine, harmine, deoxyvasicinone, deoxyvasicine, harmaline, harmol, and harmane, were isolated with their structures identified from *P. nigellastrum* Bunge, and their AChE inhibition activity was semiquantitatively evaluated by TLC-bioautographic assay [14]. Subsequently, intrigued by the interesting biological activities, Boerth and Rasapalli developed a synthetic route for nigellastrine, vasicinone, and luotonins [16]. In addition, it was also proved that the β-carboline alkaloids from *P. harmala* exhibited improvement effects on learning and memory of model dementia mice induced by aluminum [17].

The present study was undertaken to quantificationally evaluate the inhibitory activity of those alkaloids against AChE and BChE by *in vitro* Ellman method [18, 19]. Furthermore, by comparing the IC_{50} values and AChE selectivity index (SI, IC_{50} of BChE/IC_{50} of AChE) and analyzing the structure-activity relationship of those compounds investigated on selective AChE or BChE inhibition, it could provide a direction for the design and synthesis of new AChE and BChE inhibitors.

2. Materials and Methods

2.1. Chemicals and Instruments. The following chemicals were obtained from Sigma-Aldrich (USA): AChE from *Electrophorus electricus*, BChE from equine serum, acetylthiocholine iodide, butyrylthiocholine iodide, 5,5′-dithiobis-bis-nitrobenzoic acid (DTNB), L-glutathione (L-GSH), harmaline, harmine, harmane, 1,2,3,4-tetrahydroharmane-3-carboxylic acid, and galanthamine; harmalol hydrochloride dehydrate and harmol were purchased from Wako Pure Chemical Industries, Ltd. (Japan); vasicine, vasicinone, deoxyvasicinone, and deoxyvasicine were separated and purified in our laboratory from seeds of *P. harmala*, and nigellastrine I and nigellastrine II were separated and purified in our laboratory from seeds of *P. nigellastrum* (≥98% purity), and their physical and spectral data were in good agreement with the literature data [14]. The structures of those compounds investigated were showed in Figure 1.

Microplate reader (Power wave XS, Bio-Tek Instruments, Winooski, VT, USA), precise pH instrument (PB-10, sartorius, Germany), and Eyela bath SB-9 (NTT-2200, Tokyo Rikakikal Co. Ltd.) were used.

2.2. Preparation of Solutions. The enzymes AChE and BChE were dissolved in 20 mM sodium phosphate buffer (pH 7.6) to make the 3.47 unit/mL stock solution, and the solutions were stored at −80°C before use. The stock solutions of the test compounds (harmaline, harmine, harmalol, harmol, harmane, vasicine, 1,2,3,4-tetrahydroharmane-3-carboxylic acid, vasicinone, deoxyvasicinone, deoxyvasicine, nigellastrine I, nigellastrine II, and galanthamine) were prepared, respectively, by dissolving an adequate quantity of each compound in 0.2% DMSO to obtain 20 mM solutions. All the stock solutions were diluted to a series of concentrations with 20 mM sodium phosphate buffer solution (pH 7.6) before each experiment. DTNB-phosphate-ethanol reagent was prepared as follows: to 12.4 mg of DTNB dissolved in 120 mL of 96% ethanol, 80 mL of distilled water and 50 mL of 0.1 mM phosphate buffer (pH 7.6) were added [20].

2.3. In Vitro Anticholinesterase Assays. The AChE and BChE activity assay was carried out using acetylthiocholine iodide and butyrylthiocholine iodide as substrates, respectively, based on a colorimetric method, as described previously [18–20]. 10 μL of the test compounds solution in 0.2% DMSO, 79 μL of 20 mM sodium phosphate buffer (pH 7.6), and 1 μL enzyme preparation (with final concentrations: 0.087 unit/mL for AChE, or 0.035 unit/mL for BChE, and final concentrations: 1 to 500/1000 μM for compounds tested) were mixed and preincubated for 15 min. To the mixture, 10 μL substrate solution was added (final concentrations 1.5 mM for acetylthiocholine iodide, or 4 mM for butyrylthiocholine iodide) and incubated for 30 min. The reaction was stopped by adding 900 μL DTNB-phosphate-ethanol reagent. The absorption was read immediately at 412 nm on a microplate reader. The concentration of the test compound required to inhibit AChE or BChE activity by 50% (IC_{50}) was calculated using an enzyme inhibition dose response curve, with galanthamine as a positive control and L-GSH as standard to draw the standard curves. AChE SI was calculated by using the following formula: SI = IC_{50} of BChE/IC_{50} of AChE.

2.4. Data Analysis. The assays were conducted in triplicate, and all tabulated results were expressed as means ± SD, and the IC_{50} values were calculated by Boltzmann's dose response analysis using Origin 8.0 software.

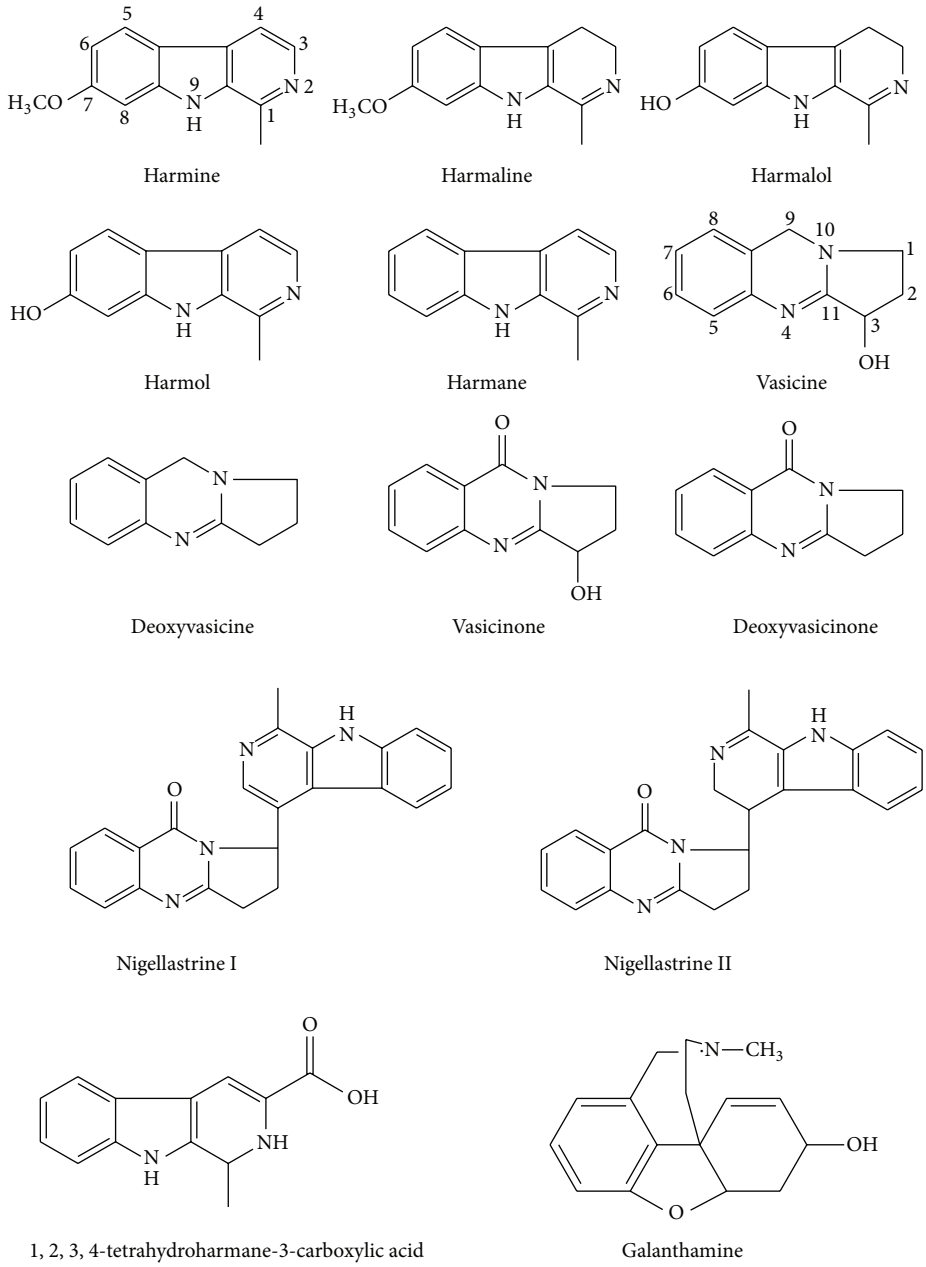


Figure 1: Structures of the compounds investigated.

3. Results

The inhibitory activities of the β-carboline and quinoline alkaloids on AChE and BChE were evaluated *in vitro* using an inhibition assay with AChE (electric eel) and BChE (equine serum) and were summarized in Table 1.

First of all, it was observed that the inhibition data of reference compounds galanthamine within the limits of the different enzyme source and assay experimental conditions are in good agreement with the literature data [21].

All tested compounds exhibited some degree of activity on both AChE and BChE, and their IC_{50} values were in the low micromolar range for at least one enzyme, with the exception of compounds deoxyvasicine and 1,2,3,4-tetrahydroharmane-3-carboxylic acid. Harmane showed the most potent inhibitory activity with IC_{50} value of 7.11 ± 2.00 μM against AChE. However, harmane had a low inhibitory activity against BChE (IC_{50} of 76.91 ± 1.57 μM). Harmine and harmaline significantly inhibited AChE activity with IC_{50} values of 9.05 ± 1.08 μM and 10.58 ± 2.01 μM, but had lower inhibitory activity against BChE with IC_{50} values of 75.07 ± 1.29 μM and 101.39 ± 1.39 μM, respectively. Vasicine exhibited strong inhibition both on AChE and BChE activities with IC_{50} values of 13.68 ± 1.25 and 2.60 ± 1.47 μM, respectively. As the main metabolites of harmaline and harmine *in vivo* [22, 23], harmalol and harmol exhibited

Table 1: Inhibitory activity (IC_{50}) and selectivity index (SI) of the compounds investigated against AChE and BChE.

Compounds	IC_{50} (μM ± SD)		SI
	AChE	BChE	
Galanthamine	1.18 ± 1.05	/	
	0.8 [21]	7.3 [21]	9.13
Harmane	7.11 ± 2.00	76.91 ± 1.57	10.82
Harmine	9.05 ± 1.08	75.07 ± 1.29	8.30
Harmaline	10.58 ± 2.01	101.39 ± 1.39	9.58
Harmol	21.58 ± 1.39	8.21 ± 3.65	0.38
Harmalol	27.88 ± 1.13	9.48 ± 2.03	0.34
Vasicine	13.68 ± 1.25	2.60 ± 1.47	0.19
Nigellastrine II	40.46 ± 1.09	9.26 ± 1.47	0.23
Nigellastrine I	70.50 ± 1.14	18.24 ± 3.23	0.26
Vasicinone	370.78 ± 1.07	—	
Deoxyvasicinone	294.44 ± 1.47	—	
Deoxyvasicine	—	—	
1,2,3,4-tetrahydroharmane-3-carboxylic acid	—	—	

IC_{50} values were determined by regression analyses and expressed as the means ± SD of three replicate determinations. SI is the AChE selectivity index defined as IC_{50} BChE/IC_{50} AChE affinity ratio.
—: no inhibitory activity.

moderate inhibitory activity against AChE with IC_{50} values of 27.88 ± 1.13 and 21.58 ± 1.39 μM and stronger inhibitory activity against BChE with IC_{50} values of 9.48 ± 2.03 and 8.21 ± 3.65 μM. Nigellastrine I and nigellastrine II had stronger inhibitory activity against BChE with IC_{50} values of 18.24 ± 3.23 and 9.26 ± 1.47 μM, but with weaker inhibitory activity against AChE with IC_{50} values of 70.50 ± 1.14 and 40.46 ± 1.09 μM.

4. Discussion

Results from previous studies showed that the alkaloids fraction containing mainly β-carboline and quinoline alkaloids from the seeds of *P. nigellastrum* exhibited potential inhibitive activity on AchE [14]. In our ongoing studies, the inhibitory activities of those individual β-carboline and quinoline alkaloids separated from genus *Peganum* on AChE and BChE were determined using the *in vitro* Ellman method. Based on the inhibitory potency and selectivity of those alkaloids on AChE and BChE, the structure-activity relationship of those alkaloids could be speculated as follows.

Amongst the series of β-carboline alkaloids (Figure 1), varying the substituent at C7 modulated the ChE inhibitory profile. The presence of an *O*-methyl substituent at C7 (harmine) led to the inhibition and selection on AChE slightly reduce with IC_{50} of 9.05 ± 1.08 μM and AChE SI of 8.30, compared to harmane with IC_{50} of 7.11 ± 2.00 μM and AChE SI of 9.13. No obvious difference was observed between the inhibitory activity of harmane and harmine against BChE. When oxidation at C7 occurs, such as harmol, the inhibition against BChE was enhanced (IC_{50} = 8.21 ± 3.65 μM) but with reduced activity on AChE (IC_{50} = 21.58 ± 1.39 μM). Therefore the hydroxyl substituent at C7 obviously reduced the selectivity for AChE inhibition. According to the above analysis, C7 could be regarded as a possible active site for β-carboline alkaloids, and varying the substituent at C7 could significantly affect the inhibition on AChE and BChE. The double bond at C3-C4 seemed to have a certain degree of influence on the inhibitory activity against BChE, as demonstrated by the IC_{50} values of harmaline and harmine. As the main *O*-demethylation metabolites of harmaline and harmine, harmalol and harmol showed moderate inhibitory potency on AChE with IC_{50} values of 27.88 ± 1.13 and 21.58 ± 1.39 μM and stronger inhibitory potency on BChE with IC_{50} values of 9.48 ± 2.03 and 8.21 ± 3.65 μM. Namely, the selective inhibition of harmalol and harmol versus AChE deduced compared with their parent compound harmaline and harmine. On the contrary, their selective inhibition against BChE enhanced.

Amongst the quinoline alkaloids (Figure 1), vasicine was identified as the potent inhibitor with IC_{50} values of 13.68 ± 1.25 μM on AChE and 2.60 ± 1.47 μM on BChE, respectively. Selective BChE inhibitors have already been reported to increase the ACh levels in the brain and to also reduce the formation of abnormal amyloid. Therefore, the discovery of potent and highly selective BChE inhibitors and/or of dual AChE/BChE inhibitors is an actively pursued goal in AD treatment drug discovery [21]. Very interestingly, although with poor selectivity, vasicine may be a very potential lead compound with the feature of dual AChE/BChE inhibitory activity.

Vasicinone and deoxyvasicinone possessing a carbonylation at C9 or dehydroxylation at C3 exhibited no much inhibitory activity on either AChE or BChE with IC_{50} values of 370.78 ± 1.07 and 294.44 ± 1.47 μM on AChE and with IC_{50} values of above 1000 μM on BChE. Those data indicated that the inhibitive potency of quinoline alkaloids on both AChE and BChE was affected by the substitution at C3 and C9. Deoxyvasicine, possessing a carbonylation reaction at C9 and dehydroxylation at C3 simultaneously, showed no inhibitory potency on both AChE and BChE with larger IC_{50} values more than 1000 μM.

As seen from Table 1, nigellastrine I and nigellastrine II (Figure 1), two dimers of quinoline (deoxyvasicinone) and β-carboline alkaloids (harmane or dehydroharmane), displayed a stronger activity on BChE inhibition and weaker activity on AChE inhibition. When compared with deoxyvasicinone, those dimers enhanced the inhibitor potency on both AChE and BChE, but more on BChE. On the other hand, by comparison with harmane, the two dimmers seemed to have certain inhibitory selectivity for BChE.

From the above analysis, harmane, harmine, and harmaline, the main active constituent in genus *Peganum*, had good selective inhibitory activities against AChE, and the oxidation of the substituent at C7 could significantly modify the inhibitory potency and selectivity for both AChE and BChE. C3 and C9 positions of quinoline alkaloids were inferred to be the active site for both AChE and BChE

inhibition. Moreover, it was found that, by the polymerization of quinoline and carboline alkaloids, the inhibitory activity declined on AChE, but increased on BChE. In the light of those findings, it could be concluded that the alkaloids from the plants of genus *Peganum* showed inhibitory activity against both AChE and BChE. In addition, those findings may provide some guidance for the design and synthesis or semisynthesis of potential inhibitors on AChE or BChE.

Conflict of Interests

The authors have declared that there is no conflict of interests.

Acknowledgments

The authors gratefully acknowledge the award from the Key Program of Joint Funds of the National Natural Science Foundation of China and Xinjiang Uygur Autonomous Region of China (no. U1130303), the National Nature Science Foundation of China (no. 81173119), the Key Project of Ministry of Science and Technology of China (2012ZX09103201-051), and the Program for Shanghai Innovative Research Team in University (2009) to Professor C.-H. Wang for the financial support of this study. The authors would also like to thank Professor Shouming Zhong for providing language help during the writing of this paper.

References

[1] J. A. Bailey and D. K. Lahiri, "A novel effect of rivastigmine on pre-synaptic proteins and neuronal viability in a neurodegeneration model of fetal rat primary cortical cultures and its implication in Alzheimer's disease," *Journal of Neurochemistry*, vol. 112, no. 4, pp. 843–853, 2010.

[2] D. Schuster, M. Spetea, M. Music et al., "Morphinans and isoquinolines: acetylcholinesterase inhibition, pharmacophore modeling, and interaction with opioid receptors," *Bioorganic & Medicinal Chemistry*, vol. 18, no. 14, pp. 5071–5080, 2010.

[3] N. H. Greig, D. K. Lahiri, and K. Sambamurti, "Butyrylcholinesterase: an important new target in Alzheimer's disease therapy," *International Psychogeriatrics*, vol. 14, supplement 1, pp. 77–91, 2002.

[4] I. Orhan, B. Şener, M. I. Choudhary, and A. Khalid, "Acetylcholinesterase and butyrylcholinesterase inhibitory activity of some Turkish medicinal plants," *Journal of Ethnopharmacology*, vol. 91, no. 1, pp. 57–60, 2004.

[5] T. Mohamed and P. P. N. Rao, "Design, synthesis and evaluation of 2,4-disubstituted pyrimidines as cholinesterase inhibitors," *Bioorganic and Medicinal Chemistry Letters*, vol. 20, no. 12, pp. 3606–3609, 2010.

[6] X. M. Cheng, T. Zhao, T. Yang, C. H. Wang, S. W. A. Bligh, and Z. T. Wang, "HPLC fingerprints combined with principal component analysis, hierarchical cluster analysis and linear discriminant analysis for the classification and differentiation of *Peganum* sp. indigenous to China," *Phytochemical Analysis*, vol. 21, no. 3, pp. 279–289, 2010.

[7] M. Kartal, M. L. Altun, and S. Kurucu, "HPLC method for the analysis of harmol, harmalol, harmine and harmaline in the seeds of *Peganum harmala* L," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 31, no. 2, pp. 263–269, 2003.

[8] B. Hemmateenejad, A. Abbaspour, H. Maghami, R. Miri, and M. R. Panjehshahin, "Partial least squares-based multivariate spectral calibration method for simultaneous determination of beta-carboline derivatives in *Peganum harmala* seed extracts," *Analytica Chimica Acta*, vol. 575, no. 2, pp. 290–299, 2006.

[9] Chinese Pharmacopoeia Committee, *Drug Standards of the Ministry of Public Health of the People's Republic of China*, Uygur Pharmaceutical Section, 1998.

[10] J. Riba, M. Valle, G. Urbano, M. Yritia, A. Morte, and M. J. Barbanoj, "Human pharmacology of ayahuasca: subjective and cardiovascular effects, monoamine metabolite excretion, and pharmacokinetics," *Journal of Pharmacology and Experimental Therapeutics*, vol. 306, no. 1, pp. 73–83, 2003.

[11] A. Tahraoui, J. El-Hilaly, Z. H. Israili, and B. Lyoussi, "Ethnopharmacological survey of plants used in the traditional treatment of hypertension and diabetes in south-eastern Morocco (Errachidia province)," *Journal of Ethnopharmacology*, vol. 110, no. 1, pp. 105–117, 2007.

[12] Z. L. Fan and X. S. Yao, "The research of constituents and pharmacology of the genus *Peganum*," *Journal of Shenyang College of Pharmacy*, vol. 9, pp. 144–151, 1992.

[13] T. Zhao, C. H. Wang, and Z. T. Wang, "Chemical constituents and pharmacologic actions of genus *Peganum*: research advances," *Journal of International Pharmaceutical Research*, vol. 37, no. 5, pp. 333–345, 2010.

[14] X. Y. Zheng, Z. J. Zhang, G. X. Chou et al., "Acetylcholinesterase inhibitive activity-guided isolation of two new alkaloids from seeds of *Peganum nigellastrum* Bunge by an in vitro TLC-bioautographic assay," *Archives of Pharmacal Research*, vol. 32, no. 9, pp. 1245–1251, 2009.

[15] X. Y. Zheng, L. Zhang, X. M. Cheng, Z. J. Zhang, C. H. Wang, and Z. T. Wang, "Identification of acetylcholinesterase inhibitors from seeds of plants of genus *Peganum* by thin-layer chromatography-bioautography," *JPC-Journal of Planar Chromatography-Modern TLC*, vol. 24, no. 6, pp. 470–474, 2011.

[16] J. A. Boerth and S. Rasapalli, "Studies toward the total synthesis of quinazoline alkaloids: vasicinone, luotonin, and nigellastrine," in *Proceedings of the 244th ACS National Meeting & Exposition*, Philadelphia, Pa, USA, August 2012, CHED-304, http://abstracts.acs.org/chem/244nm/program/view.php.

[17] J. R. Fu, L. Teng, X. Y. Dai, and F. S. Yu, "Optimization of extraction and purification of harmala alkaloids and the effects on learning and memory of model dementia mice induced by aluminum," *Chinese Traditional Patent Medicine*, vol. 33, pp. 975–979, 2011.

[18] G. L. Ellman, K. D. Courtney, V. Andres, and R. M. Featherstone, "A new and rapid colorimetric determination of acetylcholinesterase activity," *Biochemical Pharmacology*, vol. 7, no. 2, pp. 88–95, 1961.

[19] M. A. Gordon, D. E. Carpenter, H. W. Barrett, and I. B. Wilson, "Determination of the normality of cholinesterase solutions," *Analytical Biochemistry*, vol. 85, no. 2, pp. 519–527, 1978.

[20] V. Gorun, I. Proinov, V. Baltescu, G. Balaban, and O. Barzu, "Modified Ellman procedure for assay of cholinesterases in crude enzymatic preparations," *Analytical Biochemistry*, vol. 86, no. 1, pp. 324–326, 1978.

[21] B. Tasso, M. Catto, O. Nicolotti et al., "Quinolizidinyl derivatives of bi- and tricyclic systems as potent inhibitors of acetyl- and butyrylcholinesterase with potential in Alzheimer's disease," *European Journal of Medicinal Chemistry*, vol. 46, no. 6, pp. 2170–2184, 2011.

[22] T. Zhao, S. S. Zheng, B. F. Zhang et al., "Metabolic pathways of the psychotropic-carboline alkaloids, harmaline and harmine, by liquid chromatography/mass spectrometry and NMR spectroscopy," *Food Chemistry*, vol. 134, no. 2, pp. 1096–1105, 2012.

[23] A. M. Yu, J. R. Idle, K. W. Krausz, A. Küpfer, and F. J. Gonzalez, "Contribution of individual cytochrome P450 isozymes to the *O*-demethylation of the psychotropic β-carboline alkaloids harmaline and harmine," *Journal of Pharmacology and Experimental Therapeutics*, vol. 305, no. 1, pp. 315–322, 2003.

Hindawi Publishing Corporation
Journal of Chemistry
Volume 2013, Article ID 896709, 6 pages
http://dx.doi.org/10.1155/2013/896709

Research Article

The Extracts of Some Marine Invertebrates and Algae Collected off the Coast Waters of Vietnam Induce the Inhibitory Effects on the Activator Protein-1 Transcriptional Activity in JB6 Cl41 Cells

S. N. Fedorov,[1] V. B. Krasokhin,[1] L. K. Shubina,[1] S. A. Dyshlovoy,[1] N. H. Nam,[2] and C. V. Minh[2]

[1] *Laboratory of Marine Natural Products Chemistry, G.B. Elyakov Pacific Institute of Bioorganic Chemistry, Far-East Branch of the Russian Academy of Sciences, Prospect 100-let Vladivostoku 159, Vladivostok 690022, Russia*
[2] *Institute of Marine Biochemistry, Vietnam Academy of Science and Technology, Hanoi, Vietnam*

Correspondence should be addressed to S. N. Fedorov; fedorov@piboc.dvo.ru

Received 29 June 2012; Revised 6 December 2012; Accepted 19 December 2012

Academic Editor: Ana B. Martin-Diana

It has previously been shown that inhibition of the transcriptional activity of the oncogenic nuclear factor AP-1 can result in cancer prevention. Marine invertebrates and alga are a rich source of natural compounds that possess various biological activities. The inhibitory effects of the extracts of Vietnamese marine organisms in relation to the AP-1 transcriptional activity were examined by the luciferase method using JB6 Cl41 cells stably expressing a luciferase reporter gene controlled by AP-1 DNA binding sequence. As was found, 71 species of marine sponges out of 148 species studied contain inhibitors of the AP-1 transcriptional activity. Therefore, marine organisms as a source of biologically active compounds have a great potential for isolation of the new cancer preventive compounds that inhibit the oncogenic AP-1 nuclear factor.

1. Introduction

The activator protein-1 (AP-1) transcription factor is a heterodimeric complex that contains members of the JUN, FOS, ATF, and MAF protein families. AP-1 activity is induced by a plethora of physiological stimuli and regulates a wide range of cellular processes, including cell migration, proliferation, differentiation, inflammation, apoptosis and survival, transformation, and tumor promotion [1–5]. The upregulation of such AP-1 proteins as c-FOS, FOSB, and c-JUN was found to correlate with a positive effect on cell transformation [6–10]. The AP-1 has increased expression in different cancers including breast, ovarian, cervical, colorectal, lung, bladder, and many others [11–15]. Thus, AP-1 has been considered primarily to be an oncogene. Later, some of the AP-1 proteins, such as Jun-B and c-Fos, were shown to have tumor-suppressor activity both *in vitro* and *in vivo* [16, 17]. Activation of another AP-1 protein, c-Jun, is required for the induction of Fas L-mediated apoptosis in PC12 and human leukemia HL-60 cells [18, 19]. Activation of both AP-1 and NF-κB nuclear factors is necessary for DNA damaging agents- and ceramide-induced apoptosis in T lymphocytes and Jurkat T cells [20, 21]. The balance between AP-1 family members, c-Jun, and ATF-2 governs the choice between differentiation and apoptosis in PC12 cells [22]. The ultimate fate of the cells relies on the relative abundance of AP-1 complex, the composition of the AP-1 dimers, cell type, and cellular environment [23].

AP-1 transcription factor plays a role in regulatory processes essential for the specific function of cells in the immune, endocrine, nervous, cardiovascular, and other physiological systems [24, 25], and therefore it participates not only in cancer but also in such diseases as cerebral ischaemia, stroke, seizure [26, 27], psoriasis [28], and mastitis [29].

Taken together, this suggests that AP-1 is a promising target for prevention and therapy in cancer and many other diseases [30].

Many works from our laboratory and other laboratoring clearly suggested that some cancer preventive and therapeutic

compounds, including those of marine origin, can inhibit AP-1 activity, whereas other such compounds can induce it. For example, cancer preventive compounds resveratrol from grape skin, actinoporin RTX-A from sea anemones, steroidal glycoside LSG from starfishes are among agents that inhibit AP-1 activity [31–33]. On the other, hand anticancer drug vinblastine, cancer preventive flavonoids kaempferol and genistein, marine natural product 3-demethylubiquinone Q2 from ascidian *Aplidium glabrum,* and its synthetic analogs induce AP-1 activity [34–39].

In this study, the *in vitro* effects of ethanol extracts of 148 marine invertebrate species (mainly sponges) and algae on the basal AP-1-dependent transcriptional activity in mouse epidermal JB6 Cl41 cells were evaluated.

2. Experimental

2.1. Marine Invertebrates and Alga. The specimens of marine invertebrates and algae were collected off the coast of Vietnam at a depth of 2–440 m by scuba diving and trawling during the 34th scientific expedition onboard the research vessel "Academic Oparin" in May-June 2007 and were immediately extracted with ethanol. The animals and algae were identified by Dr. V. B. Krasokhin. The sponges having the same names in Table 1 belong to one and the same genus, but they may have different specific names, undetermined yet, and were collected at the different places of the coast of Vietnam. The voucher specimens are kept in the collection of G.B. Elyakov Pacific Institute of Bioorganic Chemistry of the Far Eastern Branch of the Russian Academy of Sciences, Vladivostok, Russia.

2.2. Cell Culture. The mouse epidermal JB6 Cl41 AP-1 cells were cultured in monolayers at 37C and 5% CO_2 in MEM containing 5% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin [40].

2.3. MTS Cytotoxicity Assay. Each ethanol extract of a marine invertebrate or alga was evaporated to dryness. Then, the solutions of the dry extract residue in 5% FBS/MEM with the residue concentrations of 1.0; 0.9; 0.8; 0.7; 0.6; 0.5; 0.4; 0.25; 0.125; and 0.0625 mg/mL were prepared. The effect of these solutions on JB6 Cl41 AP-1 cells viability was evaluated using MTS reduction into its formazan product [41] as described in [42]. In brief, the JB6 Cl41 AP-1 cells were cultured for 12 h in 96-well plates (6,000 per well in 0.1 mL of 5% FBS/MEM). The media was then replaced with fresh 5% FBS/MEM containing the studied substances at various concentrations in a total volume of 0.1 mL and the cells were incubated with obtained solutions for 22 h. Then, 20 μL of the MTS reagent was added into each well and MTS reduction was measured 2 h later spectrophotometrically at 492 and 690 nm as background using the Multiskan MS microplate reader (Labsystems, Finland). The solution with the first nontoxic concentration (more than 80% of living cells in the corresponding experiment) of an extract residue (see Table 1) was used in the luciferase assay (see Section 2.4).

Table 1: The effects of the extracts of the marine invertebrates and alga on AP-1-dependent transcriptional activity in JB6 Cl41 cells.

Num.*	Name[x]	Conc.[£] mg/mL	AP-1[¥]
O14-34	*Callyspongia* sp.	0.6	103
O15-34	*Oscarella* sp.	0.5	94
O16-34	*Dysidea* sp.	0.5	100
O17-34	*Mycale* sp.	0.5	**134**
O18-34	*Chondrilla* sp.	0.6	110
O19-34	*Monanchora* sp.	0.125 (0.24)**	**40**
O20-34	*Haliclona* sp.	0.6	96
O21-34	*Spirastrella* sp.	0.4	111
O22-34	Ascidian unidentified	0.8	80
O23-34	*Petrosia* sp.	0.8	**64**
O24-34	Demospongiae, unidentified	0.6	88
O25-34	*Spirastrella* sp.	0.6	**41**
O26-34	*Callyspongia* sp.	0.5	**71**
O27-34	*Spongia* sp.	0.5	98
O28-34	*Mycale* sp.	0.8	106
O29-34	*Callyspongia* sp.	0.6	101
O30-34	*Chondrilla* sp.	0.6	89
O31-34	Alga unidentified	0.5	98
O33-34	*Callyspongia* sp.	0.5	89
O34-34	*Callyspongia* sp.	0.5	**70**
O35-34	*Xestospongia* sp.	0.6	**29**
O36-34	*Spongia* sp.	0.5	**35**
O37-34	*Spirastrella* sp.	0.5	**122**
O38-34	Demospongiae, Haplosclerida, fam. unidentified	0.5	90
O41-34	*Phakellia* sp.	0.7	**59**
O42-34	*Halichondria* sp.	0.5	**74**
O43-34	*Halichondria* sp.	0.4	**125**
O44-34	*Haliclona* sp.	0.6	105
O45-34	*Halichondria* sp.	0.6	**75**
O46-34	*Haliclona* sp.	0.6	93
O47-34	Ascidian unidentified	0.6	95
O48a-34	Ascidian unidentified	0.8	**79**
O48b-34	Ascidian unidentified	0.8	96
O49-34	Echinoidea unidentified	0.8	105
O50-34	Pennatulacea unidentified	0.5	91
O52-34	Alga unidentified	1.0	95
O53-34	Alga unidentified	0.6	**76**
O54-34	Alga unidentified	0.6	89
O55-34	Alga unidentified	0.7	93
O56-34	Alga unidentified	0.5	**169**
O57-34	*Petrosia* sp.	0.5	105
O58-34	Demospongiae unidentified	0.125 (0.27)**	**19**
O59-34	*Haliclona* sp.	0.8	**74**
O60-34	*Pachastrella* sp.	0.0625 (0.17)**	**47**

Table 1: Continued.

Num.*	Name[x]	Conc.[£] mg/mL	AP-1[¥]
O61-34	*Spirastrella* sp.	0.5	**73**
O62-34	*Spongia* sp.	0.6	**71**
O63-34	*Spongia* sp.	0.5	102
O64-34	*Stelletta* sp.	0.5	**62**
O65-34	*Spirastrella* sp.	0.25 (0.35)**	**52**
O66-34	*Dysidea* sp.	0.125	**77**
O67-34	*Spongia* sp.	0.6	84
O68-34	Algae unidentified	0.5	**67**
O69-34	Opistobranchia unidentified	0.25	88
O70-34	Opistobranchia unidentified	0.125 (0.22)**	**121**
O71-34	Opistobranchia unidentified	0.8	96
O72-34	Opistobranchia unidentified	0.5	**149**
O73-34	*Spongia* sp.	0.0625 (0.1)**	**137**
O74-34	*Myxilla* sp.	0.125 (0.23)**	**14**
O75-34	*Spirastrella* sp.	0.4	108
O76-34	*Xestospongia* sp.	0.5	117
O77-34	*Stelletta* sp.	0.0625	91
O78-34	*Spongia* sp.	0.25 (0.54)**	**38**
O79-34	*Callyspongia* sp.	0.5	108
O81-34	Gorgonian gen unidentified	0.5	**35**
O82-34	Gorgonian gen unidentified	0.25	104
O83-34	Ascidia	0.6	91
O84-34	*Haliclona* sp.	0.6	**74**
O85-34	*Spirastrella* sp.	0.8	**60**
O86-34	*Halichondria* sp.	0.8	**63**
O87-34	*Spongia* sp.	0.8	**60**
O88-34	*Xestospongia* sp.	0.6	109
O89-34	*Clathria* sp.	0.8	105
O90-34	Ascidian unidentified	1.0	105
O92-34	*Spongia* sp.	0.6	93
O93-34	*Spirastrella* sp.	0.5	**124**
O94-34	*Spongia* sp.	0.6	**77**
O95-34	*Spongia* sp.	0.6	105
O98-34	Crinoidea	0.6	**71**
O99-34	Demospongiae, unidentified	0.8	93
O100-34	Demospongiae, unidentified	0.8	87
O101-34	Demospongiae, unidentified	0.25 (0.4)**	**127**
O102-34	*Spirastrella* sp.	0.5	84
O103-34	*Petrosia* sp.	0.5	**54**
O104-34	*Halichondria* sp.	0.5	104
O105-34	Alga unidentified	0.25	99
O106-34	Alga unidentified	0.0625 (0.11)**	**169**
O107-34	Alga unidentified	0.6	**144**
O115-34	Crinoidea	0.25 (0.42)**	**73**
O117-34	Nudibranchia unidentified	0.25 (0.37)**	**77**
O119-34	*Spirastrella* sp.	0.6	118

Table 1: Continued.

Num.*	Name[x]	Conc.[£] mg/mL	AP-1[¥]
O120-34	*Haliclona* sp.	0.6	88
O121-34	Demospongiae unidentified	0.6	111
O122-34	*Spongia* sp.	0.5	**38**
O123-34	*Callyspongia* sp.	0.0625	101
O124-34	*Axinella* sp.	0.6	**52**
O125-34	*Aplysina* sp.	0.6	**60**
O126-34	*Spongia* sp.	0.6	**57**
O127-34	*Spongia* sp.	0.0625 (0.16)**	**46**
O129-34	Crinoidea unidentified	0.6	84
O130-34	Crinoidea unidentified	0.5	90
O131-34	Crinoidea unidentified	0.125	83
O132-34	Nudibranchia unidentified	0.0625	84
O133-34	Ascidian unidentified	0.25 (0.37)**	**73**
O134-34	*Haliclona* sp.	0.0625	110
O135-34	*Callyspongia* sp.	0.5	**135**
O136-34	*Spongia* sp.	0.5	**79**
O137-34	Demospongiae unidentified	0.25	84
O140-34	*Spongia* sp.	0.8	**41**
O141-34	*Clathria* sp.	0.8	**63**
O145-34	*Callyspongia* sp.	0.8	**43**
O146-34	*Axinella* sp.	0.6	**33**
O149-34	*Suberites* sp.	0.8	83
O152-34	Lithistida unidentified	0.8	**67**
O153-34	*Halichondria* sp.	0.8	102
O154-34	*Penares* sp.	0.25 (0.34)**	**37**
O155-34	*Petrosia* sp.	0.8	**67**
O156-34	*Pheronema raphanus*	0.0625	90
O157-34	*Pachastrella* sp.	0.8	**49**
O158-34	Lithistida unidentified	0.8	**75**
O159-34	*Geodia* sp.	0.125	98
O160-34	Demospongiae unidentified	0.8	**55**
O162-34	*Halichondria* sp.	0.8	**60**
O163-34	*Spirastrella* sp.	0.5	101
O165-34	*Petrosia* sp.	0.8	**53**
O167-34	*Rhabdastrella globostellata*	0.5	**37**
O168-34	*Petrosia* sp.	0.125	81
O169-34	*Halichondria* sp.	0.25 (0.48)**	**36**
O170-34	*Acanthodendrilla* sp.	0.0625 (0.1)**	**53**
O171-34	*Callyspongia* sp.	0.8	**30**
O172-34	*Carteriospongia* sp.	0.0625 (0.11)**	**19**
O173-34	*Carteriospongia* sp.	0.25 (0.32)**	**76**
O174-34	*Axinella* sp.	0.25 (0.41)**	**33**
O175-34	*Halichondria* sp.	0.25 (0.33)**	**66**
O176-34	*Petrosia* sp.	0.125	110
O177-34	*Spongia* sp.	0.125 (0.19)**	**54**

Table 1: Continued.

Num.*	Name[x]	Conc.[£] mg/mL	AP-1[¥]
O178-34	*Petrosia* sp.	0.8	**56**
O179-34	*Hyalonema* sp.	0.25	80
O181-34	Porifera, Lithistida unidentified	0.5	**43**
O186-34	Porifera, Calcarea, unidentified	0.6	**55**
O187-34	*Callyspongia* sp.	0.0625	101
O188-34	*Agelas* sp.	0.8	**50**
O189-34	*Axinella* sp.	0.0625 (0.16)**	**67**
O192-34	*Agelas* sp.	0.8	**47**
O193-34	*Petrosia* sp.	0.5	**42**
O194-34	*Amphimedon* sp.	0.25 (0.39)**	**42**
O196-34	*Spongia* sp.	0.125 (0.23)**	**60**
O197-34	*Axinella* sp.	0.5	**60**
O198-34	*Plakina* sp.	0.5	**41**

*The number of the voucher specimen.
[x]Systematical name of the specimen.
[£]Concentration of the extract dry residue in the MEM.
[¥]AP-1-dependent transcriptional activity, percentage of the control.
**IC_{50}.

2.4. Luciferase Assay for AP-1-Dependent Transcriptional Activity. The effect of the studied substances on AP-1 transcriptional activation was investigated in JB6 Cl41 cells stably expressing a luciferase reporter gene controlled by an AP-1 DNA binding sequence as described earlier [43]. In brief, viable JB6 Cl41 AP-1cells (8×10^3) suspended in 100 μL of 5% FBS/MEM were added into each well of a 96-well plate. Plates were incubated overnight and the media was then replaced with fresh 5% FBS/MEM containing the extract residue at subtoxic concentration (see Section 2.3) in a total volume of 0.1 mL and the cells were incubated with the extract residue for 24 h. Then, the cells were disrupted for 1 h at room temperature with lysis buffer (0.1 M potassium phosphate buffer at pH 7.8, 1% Triton X-100, 1 mM DTT, 2 mM EDTA) and 30 μL of lysate from each well were transferred into a plate for luminescent analysis and luciferase activity was measured. Results are expressed in Table 1 as an AP-1-dependent transcriptional activity in percentage relative to untreated control cells (100%).

3. Results and Discussion

In continuation of our search for cancer preventive compounds from marine organisms [32, 33, 38, 43, 44], we studied the influence of the extracts from 148 species of marine invertebrates and algae on the AP-1-dependent transcriptional activity in mouse epidermal JB6 Cl41 cells. The JB6 Cl41 cells undergo the malignant transformation under the treatment of EGF as a promoter. The transformation involves the activation of AP-1 nuclear factor which regulates the transcription of various genes related to inflammation, proliferation and metastasis. Thus, it can be said that the extracts which show the inhibition of the AP-1-dependent transcriptional activity in JB6 Cl41 cells may contain some cancer preventive compounds. To study the effects of the ethanol soluble substances from the studied species on the AP-1 transcriptional activity, we used the luciferase assay and JB6 Cl41 AP-1 cells stably expressing a luciferase reporter gene controlled by an AP-1 DNA binding sequence. The results of the study are presented in Table 1.

We studied 148 species of marine invertebrates and algae, and there are 116 sponges, 7 ascidians, 10 species of algae, 1 species of Echinoidea, 4 species of Opistobranchia, 2 species of Nudibranchia, 2 species of Gorgonacea, 5 species of Crinoidea, 1 species of Pennatulacea. The extracts of 71 species studied showed inhibitory effects (less than 80% of activity in comparison to control) on AP-1-dependent transcriptional activity at noncytotoxic concentrations, whereas extracts of 12 species demonstrated activation (more than 120% of activity in comparison to control) of the AP-1 nuclear factor in JB6 Cl41 cells. As shown in Table 1, sponges, belonging to genera *Spongia*, *Petrosia*, *Halichondria*, *Callyspongia*, *Spirastrella*, and *Axinella* are very promising sources of the AP-1 nuclear factor inhibitors, whereas 2 species of Opistobranchia and 3 species of Algae contain activators of the AP-1 activity. We hope that our study will facilitate the further search and isolation of the AP-1 nuclear factor inhibitors and activators from marine sources.

4. Conclusion

In conclusion, the extracts of 71 species of marine organisms out of 148 species studied showed inhibitory effects on the AP-1 transcriptional activity in JB6 Cl41 AP-1 cells, and only 12 extracts demonstrated activation of the AP-1 nuclear factor. The search for natural compounds that inhibit or induce AP-1 activity may lead to the development of the new promising preventive or therapeutic drugs against various diseases including cancer.

Acknowledgments

This work was supported by Grant RFFI 11-04-0052-a, Grant NSS 546.2012.4 from the President of RF, program of presidium of RAS "Molecular and Cell Biology", and FEB RAS Grant 12-III-B-05-020. The authors are grateful to Professor Zigang Dong (Hormel Institute of Minnesota University, USA) who kindly donated the JB6 cell lines, which were used in the present study.

References

[1] R. Eferl and E. F. Wagner, "AP-1: a double-edged sword in tumorigenesis," *Nature Reviews Cancer*, vol. 3, no. 11, pp. 859–868, 2003.

[2] M. R. Young, J. J. Li, M. Rincon, and R. A. Flavell, "Transgenic mice demonstrate AP-1 (activator protein-1) transactivation is required for tumor promotion," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 17, pp. 9827–9832, 1999.

[3] E. Shaulian and M. Karin, "AP-1 as a regulator of cell life and death," *Nature Cell Biology*, vol. 4, no. 5, pp. E131–E136, 2002.

[4] L. R. Bernstein and N. H. Colburn, "AP1/jun function is differentially induced in promotion-sensitive and resistant JB6 cells," *Science*, vol. 244, no. 4904, pp. 566–569, 1989.

[5] E. Tulchinsky, "Fos family members: regulation, structure and role in oncogenic transformation," *Histology and Histopathology*, vol. 15, no. 3, pp. 921–928, 2000.

[6] W. Jochum, E. Passegué, and E. F. Wagner, "AP-1 in mouse development and tumorigenesis," *Oncogene*, vol. 20, no. 19, pp. 2401–2412, 2001.

[7] P. Angel and M. Karin, "The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation," *Biochimica et Biophysica Acta*, vol. 1072, no. 2-3, pp. 129–157, 1991.

[8] Z. Q. Wang, A. E. Grigoriadis, U. Mohle-Steinlein, and E. F. Wagner, "A novel target cell for c-fos-induced oncogenesis: development of chondrogenic tumours in embryonic stem cell chimeras," *EMBO Journal*, vol. 10, no. 9, pp. 2437–2450, 1991.

[9] Z. Dong, R. G. Watts, Y. Sun, S. N. Zhan, and N. H. Colburn, "Progressive elevation of AP-1 activity during preneoplastic-to-neoplastic progression as modeled in mouse JB6 cell variants," *International Journal of Oncology*, vol. 7, no. 2, pp. 359–364, 1995.

[10] Z. G. Dong, M. J. Birrer, R. G. Watts, and L. M. Matrisian, "Blocking of tumor promoter-induced AP-1 activity inhibits induced transformation in JB6 mouse epidermal cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 91, no. 2, pp. 609–613, 1994.

[11] J. M. Gee, J. F. Robertson, I. O. Ellis, and R. I. Nicholson, "Impact of activation of MAP kinase family members on endocrine and survival in clinical breast cancer," *European Journal of Cancer*, vol. 36, supplement 4, article 105, 2000.

[12] B. Neyns, E. Teugels, C. Bourgain, M. Birrer, and J. de Greve, "Alteration of Jun proto-oncogene status by plasmid transfection affects growth of human ovarian cancer cells," *International Journal of Cancer*, vol. 82, no. 5, pp. 687–693, 1999.

[13] B. K. Prusty and B. C. Das, "Constitutive activation of transcription factor AP-1 in cervical cancer and suppression of human papillomavirus (HPV) transcription and AP-1 activity in HeLa cells by curcumin," *International Journal of Cancer*, vol. 113, no. 6, pp. 951–960, 2005.

[14] H. Wang, M. Birkenbach, and J. Hart, "Expression of Jun family members in human colorectal adenocarcinoma," *Carcinogenesis*, vol. 21, no. 7, pp. 1313–1317, 2000.

[15] S. Linardopoulos, E. Papadakis, D. Delakas, V. Theodosiou, A. Cranidis, and D. A. Spandidos, "Human lung and bladder carcinoma tumors as compared to their adjacent normal tissue have elevated AP-1 activity associated with the retinoblastoma gene promoter," *Anticancer Research*, vol. 13, no. 1, pp. 257–262, 1993.

[16] R. Chiu, P. Angel, and M. Karin, "Jun-B differs in its biological properties from, and is a negative regulator of, c-Jun," *Cell*, vol. 59, no. 6, pp. 979–986, 1989.

[17] E. Passegué, W. Jochum, M. Schorpp-Kistner, U. Möhle-Steinlein, and E. F. Wagner, "Chronic myeloid leukemia with increased granulocyte progenitors in mice lacking JunB expression in the myeloid lineage," *Cell*, vol. 104, no. 1, pp. 21–32, 2001.

[18] H. Le-Niculescu, E. Bonfoco, Y. Kasuya, F. X. Claret, D. R. Green, and M. Karin, "Withdrawal of survival factors results in activation of the JNK pathway in neuronal cells leading to Fas ligand induction and cell death," *Molecular and Cellular Biology*, vol. 19, no. 1, pp. 751–763, 1999.

[19] T. Kondo, T. Matsuda, T. Kitano et al., "Role of c-jun expression increased by heat shock- and ceramide-activated caspase-3 in HL-60 cell apoptosis. Possible involvement of ceramide in heat shock-induced apoptosis," *The Journal of Biological Chemistry*, vol. 275, no. 11, pp. 7668–7676, 2000.

[20] S. Kasibhatla, T. Brunner, L. Genestier, F. Echeverri, A. Mahboubi, and D. R. Green, "DNA damaging agents induce expression of Fas ligand and subsequent apoptosis in T lymphocytes via the activation of NF-κB and AP-1," *Molecular Cell*, vol. 1, no. 4, pp. 543–551, 1998.

[21] S. K. Manna, N. K. Sah, and B. B. Aggarwal, "Protein tyrosine kinase p56(lck) is required for ceramide-induced but not tumor necrosis factor-induced activation of NF-κB, AP-1, JNK, and apoptosis," *The Journal of Biological Chemistry*, vol. 275, no. 18, pp. 13297–13306, 2000.

[22] S. Leppä, M. Eriksson, R. Saffrich, W. Ansorge, and D. Bohmann, "Complex functions of AP-1 transcription factors in differentiation and survival of PC12 cells," *Molecular and Cellular Biology*, vol. 21, no. 13, pp. 4369–4378, 2001.

[23] A. Gopalakrishnan and A. N. Kong, "Anticarcinogenesis by dietary phytochemicals: cytoprotection by Nrf2 in normal cells and cytotoxicity by modulation of transcription factors NF-κB and AP-1 in abnormal cancer cells," *Food and Chemical Toxicology*, vol. 46, no. 4, pp. 1257–1270, 2008.

[24] E. F. Wagner and R. Eferl, "Fos/AP-1 proteins in bone and the immune system," *Immunological Reviews*, vol. 208, pp. 126–140, 2005.

[25] Y. Yan, G.-X. Zhang, M. S. Williams et al., "TCR stimulation upregulates MS4a4B expression through induction of AP-1 transcription factor during T cell activation," *Molecular Immunology*, vol. 52, no. 2, pp. 71–78, 2012.

[26] G. Raivich and A. Behrens, "Role of the AP-1 transcription factor c-Jun in developing, adult and injured brain," *Progress in Neurobiology*, vol. 78, no. 6, pp. 347–363, 2006.

[27] M. S. Kindy, J. P. Carney, R. J. Dempsey, and J. M. Carney, "Ischemic induction of protooncogene expression in gerbil brain," *Journal of Molecular Neuroscience*, vol. 2, no. 4, pp. 217–228, 1991.

[28] R. Zenz and E. F. Wagner, "Jun signalling in the epidermis: from developmental defects to psoriasis and skin tumors," *International Journal of Biochemistry and Cell Biology*, vol. 38, no. 7, pp. 1043–1049, 2006.

[29] K. W. Kim, J. Im, J. H. Jeon, H.-G. Lee, C.-H. Yun, and S. H. Han, "*Staphylococcus aureus* induces IL-1β expression through the activation of MAP kinases and AP-1, CRE and NF-κB transcription factors in the bovine mammary gland epithelial cells," *Comparative Immunology, Microbiology and Infectious Diseases*, vol. 34, no. 4, pp. 347–354, 2011.

[30] V. D. Leaner, H. Donninger, and M. J. Birrer, "Transcription factors as targets for cancer therapy: AP-1 a potential therapeutic target," *Current Cancer Therapy Reviews*, vol. 3, no. 1, pp. 1–6, 2007.

[31] R. Yu, V. Hebbar, D. W. Kim, S. Mandlekar, J. M. Pezzuto, and A. N. T. Kong, "Resveratrol inhibits phorbol ester and UV-induced activator protein 1 activation by interfering with mitogen-activated protein kinase pathways," *Molecular Pharmacology*, vol. 60, no. 1, pp. 217–224, 2001.

[32] S. N. Fedorov, S. A. Dyshlovoy, M. M Monastyrnaya et al., "The anticancer effects of actinoporin RTX-A from the sea anemone Heteractis crispa (=Radianthus macrodactylus)," *Toxicon*, vol. 55, no. 4, pp. 811–817, 2010.

[33] S. N. Fedorov, L. K. Shubina, A. A. Kicha et al., "Proapoptotic and anticarcinogenic activities of leviusculoside G from the starfish Henricia leviuscula and probable molecular mechanism," *Natural Product Communications*, vol. 3, no. 10, pp. 1575–1580, 2008.

[34] A. Berry, M. Goodwin, C. L. Moran, and T. C. Chambers, "AP-1 activation and altered AP-1 composition in association with increased phosphorylation and expression of specific Jun and Fos family proteins induced by vinblastine in KB-3 cells," *Biochemical Pharmacology*, vol. 62, no. 5, pp. 581–591, 2001.

[35] M. Fan, M. E. Goodwin, M. J. Birrer, and T. C. Chambers, "The c-Jun NH_2-terminal protein kinase/AP-1 pathway is required for efficient apoptosis induced by vinblastine," *Cancer Research*, vol. 61, no. 11, pp. 4450–4458, 2001.

[36] A. Gopalakrishnan, C. J. Xu, S. S. Nair, C. Chen, V. Hebbar, and A. N. T. Kong, "Modulation of activator protein-1 (AP-1) and MAPK pathway by flavonoids in human prostate cancer PC3 cells," *Archives of Pharmacal Research*, vol. 29, no. 8, pp. 633–644, 2006.

[37] L. K. Shubina, S. N. Fedorov, O. S. Radchenko et al., "Desmethylubiquinone Q2 from the far-Eastern ascidian *Aplidium glabrum*: structure and synthesis," *Tetrahedron Letters*, vol. 46, no. 4, pp. 559–562, 2005.

[38] S. N. Fedorov, O. S. Radchenko, L. K. Shubina et al., "Evaluation of cancer-preventive activity and structure-activity relationships of 3-demethylubiquinone Q2, isolated from the ascidian *Aplidium glabrum*, and its synthetic analogs," *Pharmaceutical Research*, vol. 23, no. 1, pp. 70–81, 2006.

[39] S. N. Fedorov, O. S. Radchenko, L. K. Shubina et al., "Anticancer activity of 3-demethylubiquinone Q2. *In vivo* experiments and probable mechanism of action," *Anticancer Research*, vol. 28, no. 2A, pp. 927–932, 2008.

[40] C. Huang, W. Y. Ma, M. R. Young, N. Colburn, and Z. Dong, "Shortage of mitogen-activated protein kinase is responsible for resistance to AP-1 transactivation and transformation in mouse JB6 cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 1, pp. 156–161, 1998.

[41] J. A. Baltrop, T. C. Owen, A. H. Cory, and J. G. Cory, "5-(3-carboxymethoxyphenyl)-2-(4, 5-dimethylthiazolyl)-3-(4-sulfophenyl)tetrazolium, inner salt (MTS) and related analogs of 3-(4, 5-dimethylthiazolyl)-2, 5-diphenyltetrazolium bromide (MTT) reducing to purple water-soluble formazans As cell-viability indicators," *Bioorganic & Medicinal Chemistry Letters*, vol. 1, no. 11, pp. 611–614, 1991.

[42] S. N. Fedorov, L. K. Shubina, A. S. Kuzmich, and S. G. Polonik, "Antileukemic properties and structure-activity relationships of O- and S-glycosylated derivatives of juglone and related 1, 4-naphthoquinones," *Open Glycoscience*, vol. 4, pp. 1–5, 2011.

[43] S. A. Dyshlovoy, S. N. Fedorov, A. I. Kalinovsky et al., "Mycalamide a shows cytotoxic properties and prevents EGF-induced neoplastic transformation through inhibition of nuclear factors," *Marine Drugs*, vol. 10, no. 6, pp. 1212–1224, 2012.

[44] S. N. Fedorov, S. A. Dyshlovoy, L. K. Shubina, A. G. Guzii, A. S. Kuzmich, and T. N. Makarieva, "C11 cyclopentenone from the ascidian diplosoma sp. prevents epidermal growth factor-induced transformation of JB6 cells," *Drugs and Therapy Studies*, vol. 2, no. 1, article e4, 2012.

Hindawi Publishing Corporation
Journal of Chemistry
Volume 2013, Article ID 205048, 16 pages
http://dx.doi.org/10.1155/2013/205048

Review Article

Commercially Important Medicinal Plants of South Africa: A Review

R. A. Street,[1] and G. Prinsloo[2]

[1] *Traditional Medicine Laboratory, University of KwaZulu-Natal, Howard College Campus, Durban 4041, South Africa*
[2] *Department of Agriculture and Animal Health, University of South Africa (UNISA), Florida Campus, Florida 1710, South Africa*

Correspondence should be addressed to R. A. Street; street@ukzn.ac.za

Received 12 October 2012; Accepted 7 November 2012

Academic Editor: A. Hamid A. Hadi

There is a growing interest in natural plant-based remedies as a source for commercial products. Around 80% of the South African population use traditional medicines to meet their primary health care needs; however, only a few South African medicinal plants have been exploited to their full potential in terms of commercialization. The opportunity for bioprospecting of plant compounds for novel pharmaceuticals remains largely untapped. Certain renowned medicinal plants of international acclaim including buchu and rooibos are currently contributing to local enterprise; however, other exciting opportunities exist for commonly used plants which have not yet reached the international arena. This paper focuses on the key research and development contributions of 10 commercially important medicinal plants of South Africa. Traditional uses, scientific validation, commercialisation developments, as well as both potential opportunities and setbacks are discussed.

1. Introduction

Medicinal plants have been used for centuries, and numerous cultures still rely on indigenous medicinal plants for their primary health care needs [1, 2]. South Africa, a country with a strong history of traditional healing, hosts a variety of around 30,000 flowering plant species [3], accounting for almost 10% of the world's higher plant species [4]. Medicinal plants are now universally recognised as the basis for a number of critical human health, social, and economic support systems and benefits [5]. There has been a major resurgence in interest in traditionally used medicinal plants with a number of international and local initiatives actively exploring the botanical resources of southern Africa with the intention to screen indigenous plants for pharmacologically active compounds [2, 6]. Studies to determine the chemical profile and composition of medicinal plants reveal the complexity and variety of compounds all contributing to the various uses of plants in treating numerous aliments including life-threatening diseases such as HIV-AIDS, cancer, and diabetes. There is, however, a need for alternative supply of medicinal plant material as wild plants are under extreme pressure of increased demands for local and export markets. Wiersum et al. [7] reported that the intensive harvesting of wild medicinal plants due to the increasing use has in many places resulted in overexploitation and is a serious threat to biodiversity in the region. The only option for many species is cultivation at a large scale so that the wild species are maintained and become financial viable [8]. This may also contribute to a growing economy and job creation for a developing country such as South Africa by researching and exploiting the chemical treasures contained in the floral kingdom. It is estimated that up to 700,000 tonnes of plant material is consumed annually to the value of about 150 million US dollars [7]. Significant research and development opportunities exist to discover novel and useful biological activities for South African medicinal plants [9]. The aim of this paper is to review 10 prominently used South African medicinal plants. Traditional uses, scientific validation, current commercialisation developments, as well as both potential opportunities and setbacks with regards to future research and development will be discussed.

2. *Agathosma betulina* (Rutaceae)

2.1. Introduction. Agathosma betulina (Berg.) pillans is a woody shrub, commonly known as Buchu [10]. It is endemic to the Western Cape Cederburg region of South Africa and adapted to dry conditions and can be found on sunny hillsides of rocky-sandstone slopes [11]. The Khoi-San word "Buchu" is used for any fragrant plant that could be dried and powdered and therefore did not refer to a single species, but today, "Buchu" refers to the species *A. betulina* (round-leaf buchu; "bergboegoe"; short buchu) and *Agathosma crenulata* (oval-leaf buchu; "anysboegoe"; long-leaf buchu) [12]. Buchu plant material processed into buchu oil, buchu extract, dried buchu leaves, buchu water, and powdered buchu is not only used for medicinal and cosmetic purposes, but also as flavouring agents in the food industry [10]. Buchu is highly sought after globally for its essential oils, especially since it contains high diosphenol and low pulegone content [12].

2.2. Traditional Uses. Buchu is an important plant in the Khoi-San tradition [4] and still enjoys a great reputation as a general health tonic, diuretic, and mild urinary antiseptic [10]. The essential oil is a valuable flavour product and is probably responsible for the antispasmodic, antiseptic, and diuretic activities [13, 14]. Some medicinal uses of *A. betulina* include treatment as an antispasmodic, antipyretic, liniment, cough remedy, cold and flu remedy, diuretic, treatment of kidney and urinary tract infections, haematuria, prostatitis, cholera, stomach ailments, rheumatism, gout, bruises, calculus, and an antiseptic [15, 16]. Tinctures of buchu have a great reputation as general health tonics, treatment for stomach aches, use as aromatic bitters, diuretics, and mild urinary antiseptics [4]. The San lubricated their bodies with aromatic plants mixed with fat to keep their skin soft and moist in the desert climate. The lubrication also served as an antibacterial and antifungal protectant, acted as an insect repellent, a deodorant, and to promote the general well-being of the body by uptake of the aromatic substances through the skin [10].

2.3. Phytochemistry and Biological Activity. The composition of buchu has been well studied, and numerous compounds have been identified. The compounds identified in *A. betulina* mostly focused on the volatile fractions of the plant and included limonene, menthone, diosphenol, *l*-pulegone [17] with isomenthone, and diosphenol as the major volatile compounds [18, 19] responsible for the distinctive flavour but probably also for antispasmodic, antiseptic, and diuretic activities [12–14, 20]. The commercially important sulphur-containing compounds are a characteristic of *A. betulina* (cis and trans-8-mercapto-p-methan-3-one), and although these occur in small amounts, they are responsible for the characteristic organoleptic properties of the oil [21]. No alkaloids have been found in *A. betulina* [12]. Animicrobial activity, antioxidant activity, and anti-inflammatory activity have been demonstrated in *A. betulina* but showed weak antioxidant activity, with extracts having IC_{50} values >100 μg/mL [12]. Extracts were active against all pathogens tested in a study by Moolla et al. [12] with greater activity towards the Gram-positive bacteria than the Gram-negative bacteria.

2.4. Toxicity. The extract of *A. betulina* was investigated by Moolla et al. [12] for toxicity and was found not to be toxic at IC_{50} values of up to 100 μg/mL, but with serial dilution, different degrees of cellular inhibition were displayed, and the samples proved to be toxic in a dose dependant manner.

2.5. Commercialisation and Conservation. Buchu has been wild harvested at least since 1820 and cultivated since the 1970s or perhaps even earlier [20]. Until 1995, the only reliable source of buchu material was from plants growing in the wild. Diminished habitat, increased commercial demand, destructive wild harvesting practices, and growing genetic vulnerability to natural disasters are all serious threats to sustainable supply of buchu and survival of genetic material in the wild [22]. Recent efforts in crop development have resulted in buchu now being a viable option for small-scale farming [20]. However, cultivation of buchu as a commercial crop is not yet developed, and several research questions need to be addressed to supply the demand of buchu with cultivated material [22]. Incorrect cutting procedures, over harvesting, and poor harvesting regimes inhibit buchu plants from producing seed for its next generation. Frequent fires, invasive alien plants, structural development, and increasing agricultural practices are all contributing to declining populations of buchu in their natural environment [22]. Buchu is a high value crop where fresh plant material currently sells for R35/kg. Approximately 20,000 plants can be planted per hectare, and the oil yield is approximately one percent, depending on the water status of the plant at harvesting. Global production of buchu essential oil falls into the category of 1–50 tonnes and is classified as one of the top 20 in this class [23]. Moolla and Viljoen [22] reported that the oil sells on the international market for about 700 Euros per kilogram, and buchu seed sells for R20 000 per kilogram, and Lubbe and Verpoorte [23] reported the price of buchu at $56/kg for cultivated material. These economic figures make cultivation of buchu very attractive and an excellent crop to provide an income for small-scale farmers with small quantities of land available for cultivation. Buchu is harvested by hand, and since no mechanical equipment is used, it can significantly contribute to job creation and sustainable income generation in South Africa [22].

3. *Aloe ferox* (Asphodelaceae)

3.1. Introduction. Aloe ferox (Mill.), commonly known as Cape Aloe or bitter Aloe, is a single-stemmed, robust aloe reaching a height of around 10 m tall. The broad, succulent leaves reach around 1 m in length. "Ferox" meaning "ferocious" refers to the thorny sharp red-brownish spines on the leaves [24]. Bright red or orange flowers are produced between May and August, and the rich nectar attracts numerous insects and birds [25]. Although *A. ferox* has an extensive

distribution in South Africa, it is largely concentrated along the eastern parts [26].

3.2. Traditional Uses. *Aloe ferox* is highly regarded for its laxative properties [25]. The leaf is traditionally used for skin and hair treatments [15]. Cut leaves are applied directly to burns, insect bites, sores, and sunburn [25]. Leaf and stem decoctions are used as emetics, whilst leaves and roots are boiled in water and taken for hypertension and stress [27]. Traditional preparations are also used for arthritis, conjunctivitis, toothaches, sinusitis, and stomach pains [25].

3.3. Phytochemistry and Biological Activity. The Cape Aloe leaf can be divided into two main portions: the external green rind and the internal clear pulp. The major components in fresh aloe bitters of the green rind are aloeresin A, aloein, and aloin, representing 70%–97% of total dry weight in a ratio of approximately 4 : 3 : 2, respectively [28]. The phytochemistry of *A. ferox* has been comprehensively studied, and numerous classes of compounds have been isolated including chromones, anthraquinones, anthrones, anthrone-*C*-glycosides, and other phenolic compounds (for a detailed review, see Chen et al. [24]). A study on the chemical composition of volatile constituents from the leaves of *A. ferox* identified 21 compounds, representing more than 99.99% of the essential oil [29]. The most abundant components of the volatile compounds included 3, 6 octatriene (23.86%), 3-cyclohexane-1-hetanol (7.31%), bornylene (5.24%), 1, 3-cyclopentadiene (4.07%), and 5-methyl-3-heptanol (3.92%). Scientific studies have verified numerous traditional uses such as laxative effects [30] and antioxidant [31], anti-inflammatory [32, 33], antimicrobial [34], anthelmintic [35], and anticancer [36] properties. Using the low-dose streptozotocin diabetes rat model, recent studies have shown promise of *A. ferox* in treating diabetes [37].

3.4. Toxicity. Preparation of *A. ferox* is generally considered safe; however, adverse effects such as hypersensitivity have been described [24]. It is advised that preparations should be avoided during pregnancy [38].

3.5. Commercialization and Conservation. *Aloe ferox* was first exported to Europe in 1761 and today is considered South Africa's main wild-harvested commercially traded species [24]. The selection of high-yielding sources, with total aloin levels above 25%, is recommended for commercial cultivation [39]. Apart from its medicinal applications, owing to the gels richness in minerals, amino acids, vitamins, and trace elements, it is extensively used in the cosmetic industry too [40]. The industry yields some 400 tonnes of dried leaf exudate per annum from the leaves of around 10 million plants [41] with the worth to small-scale rural harvesters in the region of R12–15 million per year [42]. Although there is a high harvesting pressure on this species, leaf harvesting can be done sustainably with minimal risk to plant survival [25]. None the less, possible size classes on which harvesting should be discouraged include the small, prereproductive individuals and individuals taller than 2.5 m which are too tall to be harvested by hand [42]. In South Africa, the *A. ferox* industry is covered by topical legislative developments in bioprospecting, access, and benefit sharing regulations within the National Environmental Management Biodiversity Act [43]. As the industry expands, the potential of the *A. ferox* to bring economic gains to underprivileged rural areas has been acknowledged, but success will depend on effective policy governing access to the resource and vigilant sustainability management [43].

4. *Aspalathus linearis* (Fabaceae)

4.1. Introduction. *Aspalathus linearis* (Burm.f.) Dahlg. produces the well-known herbal tea, called rooibos, also known as the long-life tea in Africa [44], and is endemic to the South African fynbos species [45]. Rooibos tea has become a popular herbal tea with a growing reputation of having important health benefits, including antispasmodic, antioxidant, antiageing, and antieczema activities [4, 10, 15, 45, 46]. The characteristic sensory quality of rooibos infusions is described as a mixture of honey, woody, and herbal-floral flavours with a slightly sweet taste and a subtle astringent mouthfeel [47]. Other flavours include caramel and a sweet-associated fruity flavour and sometimes off-taints such as grassy, hay-like, and musty/dusty notes and a slightly bitter or sour taste in poor quality tea [47]. Rooibos is exported to the East and Europe [48] and is currently sold in more than 37 countries with Germany, The Netherlands, the United Kingdom, Japan, and the United States of America representing 86% of the export market in 2010. The popularity of rooibos can be ascribed to its health-promoting properties, no harmful stimulants, as well as the lower tannin content when compared to black tea (*Camellia sinensis*) [10, 49].

4.2. Traditional Uses. Rooibos is a traditional beverage of the Khoi-descended people of the Cape. The Khoi crudely processed it by cutting, bruising, fermenting, and drying the twigs and leaves [10]. The first person to realise the commercial potential of rooibos as a herbal tea was Benjamin Ginsberg, a merchant of Clanwilliam, who started marketing it in 1904. It was, however, only by 1930 that the agricultural value of rooibos was recognised by a medical practitioner and nature lover, P. Le Fras Nortier of Clanwilliam [45]. African women take rooibos during pregnancy to relieve heartburn and nausea; they take it for its iron content, and they give it to their babies for colic relief and as a milk substitute for infants [44]. Rooibos is gaining acclaim for its vitamin, mineral, and antioxidant content and taste. It has become popular as a health drink because of the absence of caffeine or other stimulants and its properties to relieve digestive troubles and antispasmodic properties [10, 44].

4.3. Phytochemistry and Biological Activity. Due to the commercial importance and health benefits ascribed to rooibos, the plant has been significantly studied especially related to its antispasmodic, antioxidant, antiageing, and antieczema activities [4, 10, 15, 45, 46]. Rooibos is known as a caffeine-free herbal tea, although traces of the alkaloid sparteine have

been reported [50]. Rooibos contains two unique phenolic compounds, namely, aspalathin [51], a dihydrochalcone C-glucoside, and aspalalinin [52], a cyclic dihydrochalcone. Aspalathin and nothofagin, the major rooibos flavonoids, anti-spasmodic only showed moderate antimutagenic effects. The flavonoids quercetin and isoquercitrin have shown mutagenic and promutagenic activity as well as comutagenic and antimutagenic properties in a study by Snijman et al. [53]. Furthermore, aspalathin and nothofagin and their structural flavonoid analogues displayed moderate antimutagenic properties. Compounds such as luteolin and chrysoeriol exhibit similar activity to those of the green tea flavonoid (−) epigallocatechin gallate (EGCG). Rooibos is well known for its antioxidant activity which also relates to its hepatoprotective properties [46] and immune modulating effect in stimulating antibody production [54]. Antimicrobial effects against several bacteria have been demonstrated in a study by Scheepers et al. [55]. Rooibos consumption has also shown to be relevant to heart disease as it significantly improves the lipid profile as well as redox status by modulating the serum lipid profile significantly by decreasing the triacylglycerol and LDL-cholesterol levels and increasing the HDL-cholesterol level [56]. Very little information is available on the structure of the tannins from rooibos tea, but the dimer, procyanidin B3, the trimer, bisfisetinidol bisfisetinidol-(4β,6:4β,8)-catechin, and a pentamer have been identified [57]. Other major phenolic compounds present in rooibos include flavones (orientin, isoorientin, vitexin, isovitexin, luteolin, and chrysoeriol), flavanones (dihydro-orientin, dihydro-isoorientin, and hemiphlorin), and flavonols (quercetin, hyperoside, isoquercitrin, and rutin) [52, 57–60]. Phenolic acids, lignans, flavone diglycosides, (+)-catechin, a phenylpyruvic acid glycoside, the flavonol quercetin-3-O-robinobioside, and the coumarins, esculetin and esculin have also been identified [52, 61–64].

4.4. Toxicity. Since rooibos has gained popularity as a health beverage, no toxicological studies have been done. A number of studies have addressed aspects of safety and toxicity of rooibos [46, 49]. Although some compounds in rooibos have been shown to contain mutagenic properties [53], it is, however, very unlikely that the mutagenic effect of rooibos would be relevant to tea drinkers when considering the quantities consumed [46]. It has also been demonstrated that chronic consumption of aqueous extracts of unfermented and fermented rooibos by rats over a period of 10 weeks did not cause any adverse effects in the liver and kidney [46].

4.5. Commercialisation and Conservation. Rooibos tea is a traditional beverage of the Khoi-descended people of the Clanwilliam region in the Cape and is one of only a few indigenous plants of South Africa that have become an important commercial crop [10]. The first attempts at rooibos cultivation were in the early 1930s, but it was only after World War II that commercial cultivation started in earnest. Cultivation occurs mainly in the Cederberg mountain region but extends to areas as far as Darling and Nieuwoudtville [10]. Seedlings are used to propagate the plants, leading to large phonological, and genetic variation in the cultivated plants [46]. It has also been demonstrated by Koch et al. [47] in the sensory attributes among rooibos samples from different regions. The plants are topped to a height of 30 cm after 8 months to stimulate branching, but full production is only realised after 3 years [46]. Growing participation of other farmers in rooibos production and increased demand over the years expanded the area under cultivation to 36,000 ha with production mainly concentrated in the Clanwilliam area [10]. Only the so called red type or Rocklands type is cultivated, but there is a renewed interest in harvesting some of the wild types for niche markets and fair trade networks [10]. The red type is divided into the selected, improved Nortier type and the Cederberg type (wild-growing). The latter type has broader and coarser leaves than the Nortier type [65]. Wild rooibos regained prominence as a source of income in recent years. Small-scale producer organisations in the Cederberg (Wupperthal) and Southern Bokkeveld (Heiveld) supply wild-harvested rooibos under organic and fair trade certification to niche markets abroad [66]. Wild rooibos comprises about 2%–5% of the annual production of 40 tons rooibos by the Heiveld Cooperative [67]. Wupperthal's production of wild-harvested rooibos is approximately 10 tons [68]. The market for rooibos showed a steady increase over the years from 5633 tons in 2010 compared to 750 tons in 1993 [69]. By 2010, sales of rooibos have reached more than 5000 tons with an estimated retail sales value of R429 million [46]. Annual production from plantations has reached 20000 tonnes per annum, and export volumes now exceed local consumption [20].

5. *Harpagophytum procumbens* (Pedaliaceae)

5.1. Introduction. Harpagophytum procumbens (DC. ex Meisn.) is a perennial herb native to countries within the southern African continent such as Botswana, Namibia, and South Africa [70]. The weedy plant has grey/green leaves with yellow and violet or uniformly dark tubular flowers [10]. The vernacular name, devils claw, is derived from its fruit which are covered in small claw-like protrusions [70]. Originally, these were considered an irritation to beef farmers as the fruit can cripple an animal if lodged on its feet, but this also encourages seed dispersal [71].

5.2. Traditional Uses. The part used medicinally is the water-storing secondary tuberous roots which are formed in order to survive the dry seasons encountered in the sub-Saharan regions [70]. The plant is commonly used to treat rheumatism, arthritis, diabetes, gastrointestinal, disturbances, menstrual difficulties, neuralgia, headache, heartburn, and gout [10, 72] in the form of infusions, decoctions, tinctures, powders, and extracts [71]. While infusions and decoctions are the most central traditional methods of preparation, the alleged main active principles in the plant are prone to hydrolysis [71].

5.3. Phytochemistry and Biological Activity. The activity of *H. procumbens* has been attributed to iridoid glycosides, mainly harpagoside (0.5% to 3%), with lesser amounts of

harpagide and procumbide [10]. Though the phytochemistry of this plant has been well researched, there is a scantiness of information on the additive or synergistic effects of the chief compounds [71]. Scientific studies of the biological activity of *H. procumbens* affirm traditional uses including analgesic, antioxidant, antidiabetic, antiepileptic, and antimalarial properties—for an extensive up-to-date review, see Mncwangi et al. [71].

The therapeutic effect appears to be derived only for the whole extract as separate components show little to no advantageous effects [40]. From numerous *in vitro* and *ex vivo* studies, it is clear that *H. procumbens* has noteworthy effects on numerous proinflammatory markers [73, 74]; however, the exact mechanism(s) of action responsible for the anti-inflammatory/analgesic actions remain elusive [70, 71]. A recent hypothesis is that *H. procumbens* inhibits induction of proinflammatory gene expression, perchance by blocking the AP-1 pathway [75]. The hydrolysed products of the harpagide and harpagoside have significant anti-inflammatory activity when compared to the unhydrolysed compounds [76]. Dosage of *H. procumbens* root extract used in clinical trials in low back pain typically ranges from 2 to 4.5 mg daily corresponding to around 30–100 mg harpogaside [72].

5.4. Toxicity. The clinical trials conducted to date offer support regarding *H. procumbens* as a beneficial treatment for the alleviation of pain and improvement of mobility in a range of musculoskeletal conditions, either as a singular therapy or as an adjunct [70]. However, a systematic review on the safety of *Harpagophytum* preparations for osteoarthritic and low back pain revealed adverse events (mainly gastrointestinal) during treatment [77]. To date, there are no reports on negative interactions with conventional drugs ordinarily used for rheumatoid arthrosic conditions thereby supporting the utilization of *H. procumbens* as an adjunct therapy for these complaints [70]. Although there are no reports on chronic toxicity, more safety data are urgently needed to assure the safety of *Harpagophytum* use, particularly long term [77]. In light of possible cardio activity, it is not recommended for long periods of time [72]. Owing to the lack of data regarding the effect of *H. procumbens* during pregnancy and lactation, it should be avoided [72].

5.5. Commercialization and Conservation. Harpagophytum procumbens is traded under the pharmaceutical name of Harpagophyti radix [78]. *Harpagophytum* spp. products are mostly registered as a herbal medicine in France and Germany or as a food supplement in the United Kingdom, The Netherlands, the United States, and the Far East. In the European Pharmacopoeia, common standards for the composition and preparation of *H. procumbens* substances are described with the aim of quality assurance [78]. The harpagoside content of commercial extracts ranges from 0.8% to 2.3% [72]. The total trade for all southern African countries is about 700 tonnes per annum, with the largest portion of the world's supply emanating from Namibia [79]. Main importing nations are European counties, Japan, the USA, and Venezuela [40]. The governments of the main range states (Namibia, Botswana, and South Africa) have developed policies and regulations in an attempt to protect the species and to determine a sustainable harvest [78].

6. *Hypoxis hemerocallidea* (Hypoxidaceae)

6.1. Introduction. Hypoxis hemerocallidea, commonly known as African potato, Ilabatheka, Inkomfe, sterretjie, or starflowers, is a genus of the family Hypoxidaceae [80, 81]. In South Africa, the genus is distributed in five provinces, namely, KwaZulu-Natal, Eastern Cape, Mpumalanga, Limpopo, and Gauteng but is also found in Lesotho, Swaziland, Mozambique, and Zimbabwe [81–83]. Medicinally, the African potato has been used by different cultures for many ailments such as bad dreams, cardiac diseases, impotency, apprehension, barrenness, and intestinal parasites, and today it is even used for cancer, headaches, dizziness, as an immune booster, testicular cancer, prostate hypertrophy, burns, and ulcers [82].

6.2. Traditional Uses. A number of medicinal properties have been assigned to the use of *H. hemerocallidea* and is therefore traded in large numbers at medicinal markets. *Hypoxis* has been used traditionally for benign protatic hypertrophy and urinary tract infections and as a laxative and vermifuge [10]. The corm of the plant has been used in folk medicine to treat a variety of diseases which include common cold, flu, hypertension, adult-onset diabetes, psoriasis, urinary infections, testicular tumours, prostate hypertrophy, HIV/AIDS and some central nervous system disorders, anticancer, antidiabetic, antimicrobial, antioxidant, and anti-inflammatory treatment. The cultural uses of *Hypoxis* include charm against lightning, thunder, and storms and also as an emetic for fearful dreams. The decoction of other species of *Hypoxis* is recorded as being used as a steam bath against venereal diseases such as lice [10, 84–86]. Other traditional uses include treatment of ailments such as asthma, tuberculosis, urinary tract infection, headache, heart weakness, dizziness, duodenal and gastric ulcers, internal cancer tumours, and some central nervous system ailments, specially epilepsy [10, 38, 87].

6.3. Phytochemistry and Biological Activity. The uses of this plant can probably be attributed to a few of the medicinal compounds found in the plant of which hypoxoside, sitosterol, and its aglycone derivative rooperol are probably the most well-known compounds [38]. Hypoxoside is the trivial name for (E)-1,5 bis(4′-B-D-glucopyranossyloxy-3′-hydroxyphenyl)pent-1-en-4-yne which is a norlignan diglucoside isolated from the rootstock of the family Hypoxidaceae. In addition to hypoxoside and rooperol, the rootstocks are reported to contain β-sitosterols, sterol, monterpene glycosides, stanols, and stigmastanols [84]. The result of the chemical analysis of *H. hemerocallidea* showed that the species have different classes of secondary metabolites, namely, glycosides, polyphenols, saponnins, steroids, and tannins [88]. The rootstock of *H. hemerocallidea* has yielded three cytokinins, identified as zeatin, zeatin riboside, and zeatin glucoside [38]. The high concentration of phytosterols

such as β-sitosterols has proven to be effective against benign prostate hypertrophy [89], and a strong decoction of the rootstocks is also used as purgatives [87]. Rooperol may be obtained by treating hypoxoside with a β-glucosidase to remove the attached glucose groups. β-glucosidase is an enzyme found predominantly in the gastrointestinal tract [87]. According to Ojewole [85], the findings of the experimental animal study indicate that *H. hemerocallidea* corm aqueous extract possesses anti-inflammatory and antidiabetic properties in the mammalian laboratories animal models used. The rootstock may be useful in the management of adult onset, noninsulin-dependent, type 2, diabetes mellitus [90].

6.4. Toxicity. It was shown conclusively that Hypoxis extracts (45% hypoxoside) are not toxic [84]. In a later paper, the possible treatment of certain malignancies and HIV-infection with hypoxoside as a putative, nontoxic prodrug is mentioned [91]. The extracts and its bioactive compound hypoxoside have not exhibited toxicity in several clinical or toxicity studies [84, 91].

6.5. Commercialisation and Conservation. *H. hemerocallidea* is an important plant species in traditional medicine in southern Africa, and the use of the corms is so popular that the species is threatened by overharvesting [92]. *H. hemerocallidea* was found to be one of the top 10 most frequently sold plant species with approximately 11000 kg/year sold valued at R322 500 [93]. The importance of this plant in traditional medicine and the potential importance in the pharmaceutical industry necessitate an efficient means of propagating these plants [94]. Apart from traditional plant usage, it is also exported to the East and Europe [95], which contributes to the need for sustainable supply. The plant grows easily and in a wide range of environments but is extremely difficult to propagate in large quantities. A considerable amount of work has already been done on tissue culture and seed germination [94, 96–99] but without resolving a method which can supply the market with the material needed at a reasonable cost.

7. *Merwilla natalensis* (Hyacinthaceae)

7.1. Introduction. *Merwilla natalensis* (Planch.) Speta, also known as *Scilla natalensis* planch, *Merwilla plumbea* (Lindl.) is indigenous to South Africa and is distributed throughout the eastern part of South Africa including Lesotho, KwaZulu-Natal, Free-State, Swaziland, and the Northern Province [100]. *Merwilla natalensis* is traditionally known as "inguduza" which means "searching the body for the cause of the ailment" [101]. The extracts of the bulb are known for their antibacterial, anthelmintic, anti-inflammatory, and antischistosomal medicinal activity and use as ointment for wounds, scarifications, as a laxative, and as an enema [10]. *Merwilla* is sought after for its traditional and medicinal properties to such an extent that there is a risk of extinction [102].

7.2. Traditional Uses. *Merwilla natalensis* is used traditionally by various cultures such as the Zulu, Tswana, Swati, and Sotho speaking people in South Africa. The bulb is used traditionally in Africa amongst others for gastrointestinal ailments, sprains and fractures, tumours (cancerous), menstrual pains, infertility, and to support the process in woman to deliver their babies [103]. Cooked bulbs are eaten together with other food by the southern Sotho ethnic people that believed that it treats "internal tumors" [104]. Tswana ethnic people rub the powdered bulb onto their back joints to improve their power and spirit. It has also been administered to treat internal tumors, lumps, or other cancerous or noncancerous swellings by purgatives or an enema [10, 15]. Swati ethnic people use boiled water extracts of the bulbs for the treatment of sprains, boils, fractures, and veld sores [104]. Gynaecologically, extracts of *Merwilla* assist in delivery and relief of menstrual pains (amenorrhea). A decoction of the bulbs is known to be taken as enemas for the fertility of women and to boost libido and potency of men. The decoctions are usually made by boiling the bulb in milk or water [10, 104]. Although the sap released from the plant can burn the skin of humans, ointment is prepared via the decoction method from fresh bulb scales as well as burnt scales to treat external wounds, boils, abscesses, and fractures. The ashes of the burnt plant are rubbed into cuts and scratches for relief [105].

7.3. Phytochemistry and Biological Activity. Numerous studies support the use of the plant for treatment of the previously mentioned ailments. The bulb extracts have shown antibacterial, antischistosomal, anti-inflammatory, and anthelmintic activity in studies by Sparg et al. [100, 103]. The ethanol extracts showed the best activity against several bacteria [103], and the leaf extracts show anticandidal activity with a MIC of <1 mg/mL only in spring and autumn, whereas the bulb extracts have a MIC of >1 mg/mL in all seasons [106]. The pharmacological active compounds from the bulbs of *Merwilla* can act against gastrointestinal ailments such as diarrhoea, intestinal worms, constipation, nausea, and stomach aches [38]. Good activity against nematodes of *M. natalensis* extracts was observed where the ethanolic extract killed 50% of the nematodes, dichloromethane, and hexane extracts (40%), and water extracts (30%) [103]. *M. natalensis* is considered as a soap plant due to its content of saponins which produce foam once in contact with an aqueous solution. The phytochemical screening of *M. natalensis* bulbs revealed the presence of saponins and bufadienolides within the bulbs [103]. Hyacinthaceae species are known to contain cardiac glycosides being confirmed by a study by Kellermen et al. [107]. Antimony (III) chloride ($SbCl_3$) reagent was used to identify cardiac glycosides, specifically the bufadienolide type with a high probability of Proscillaridin A to be present in *M. natalensis* bulbs [103]. Most of the Hyacinthaceae species also contain homoisoflavanones, which possess antibacterial activity [108, 109]. Homoisoflavanones were also detected in *M. natalensis* by Crouch et al. [110], and this is one of the components that are responsible for the anti-inflammatory properties [103]. The dichloromethane and hexane extracts of *M. natalensis* resulted in good inhibition against both COX-1 and COX-2, but the aqueous and ethanolic extracts showed no noteworthy activity [103]. In the

study by Du Toit et al. [109], homoisoflavonones show good activity against *Staphylococcus aureus*. The simple structure of homoisoflavanones consists of chromane, chromanone, or chromone together with a benzylidene or benzyl group that is attached to the third position of a 16-carbon skeleton [109, 111]. Poor antibacterial activity against both Gram-positive and Gram-negative bacteria was, however, shown with *M. natalensis* extracts [103]. The storage of the bulb did not have any negative influence on the antibacterial activity of the extracts but actually increased the activity [112]. Sparg et al. [103] confirmed that there are no alkaloids present in *M. natalensis*. Other chemical compounds that are present in *M. natalensis* are five types of nortriterpenoids, one bisnortriterpenoids, and one trisnorterpenoid. Ethanolic extracts had the highest inhibitory effect against nematodes in the anthelmintic assays, and the aqueous extracts of both dry and fresh bulbs showed extremely good activity against *Schistosoma haematobium* [103].

7.4. Toxicity. With the powerful components that are present in the plant, it can be used as a medicinal plant, but precaution must be taken as it can be toxic. Since cytotoxicity was found, it was suggested that care should be taken when used internally [103]. The toxic cardiac glycoside transvaalin was showed to be the same compound as scillaren A which is found in similar species related to *M. natalensis* [48] but should still be demonstrated in *M. natalensis* [10]. No genotoxic effects were found, however, in a study by Elgorashi et al. [113], using the Ames and VITOTOX tests. Genotoxicity was, however, found by Fennell et al. [114], using the micronucleus test where structural and chromosome aberrations were observed. It was also found by Sparg et al. [103] that *M. natalansis* is extremely toxic to VK cells with morphological changes, cell death, and severe cytotoxicity observed at all concentrations tested.

7.5. Commercialisation and Conservation. Merwilla natalensis is classified as a vulnerable species and therefore included in the red data list [115]. For the reason that *M. natalensis* bulbs are in high demand to support the use of medicinal and traditional use, *M. natalensis*is is recommended for cultivation [116]. In order to protect the plant from extinction, the supply of *Merwilla* plants need to be higher than the demand. Slow-growing bulbous and tuberous plants, which are frequently used in traditional Zulu medicine, represent another group of plants that are particularly threatened by overexploitation and recognized by the healers as becoming scarce [117]. Again, the main problem is the destructive harvesting of the underground parts of these plants or even the plant as a whole [118]. The bulbs of *Merwilla* take 15 years to get to its most preferable size [101]. *Merwilla natalensis* is one of the cheapest plants per kilogram (R 6.50/kg) [117], and in 2006, 2.1 million bulbs of *Merwilla* were sold in and around Johannesburg and Durban to the value of R3.87 million [101]. It is extremely easy to cultivate since the plant produces numerous seeds which can be easily germinated with a high survival of the seedlings. Seed propagation is therefore preferred as the most effective method for cultivation [119]. Care should be taken in ensuring quality of cultivated material as reduction in some biological activity was found when cultivated under irrigation [114].

8. *Pelargonium sidoides* (Geraniaceae)

8.1. Introduction. The taxonomic classification of *Pelargonium* species is noted with some difficulty hence the numerous revisions in the past. Nonetheless, the genus *Pelargonium* is highly esteemed by traditional healers for its therapeutic and palliative effects in the treatment of gastrointestinal disorders [120]. *Pelargonium sidoides* DC. is native to the coastline regions of South Africa and Lesotho [2]. The small perennial herb has long-stalked leaves which are slightly aromatic, heart-shaped, and velvety. The characteristic dark, reddish-purple tubular flowers are present throughout most of the year but occur most frequently from late spring to summer [2, 121]. In the early 20th century, Charles Henry Stevens was treated in South Africa by a traditional health practitioner who claimed that he cured his tuberculosis with a natural remedy containing *P. sidoides*. "Umckaloabo" is the name used by Stevens for his tuberculosis medicine, a name that still persists today. The term "Umckaloabo" may actually be an origination of Stevens, based on South African languages, intended to create an enigmatic image for his remedy to increase its marketability. In the first half of the 20th century, a product made from the root was used in Europe to treat tuberculosis [2]. A summary of the fascinating history of the commercial development of "Umckaloabo" in Europe is presented in Brendler and van Wyk [122].

8.2. Traditional Uses. Although a modern aqueous-ethanolic formulation of *P. sidoides* extracts (EPs 7630) is an efficacious treatment for disorders of the respiratory tract, the traditional uses mainly involve disorders of the gastrointestinal tract [120, 122]. The fleshy, bright red rhizomes or tubers of *P. sidoides* are used predominantly to treat diarrhoea and dysentery [15, 122]. In fact tannin-containing plant remedies are traditionally used to treat diarrhea [122]. *Kalwerbossie* is a local name used for *P. sidoides* because the plants were traditionally used as a remedy for worms in calves (kalwers) [122]. In the eastern Cape, the crushed roots of *P. sidoides* are mixed with water after which a teaspoon is given orally to infants with upset stomachs [123].

8.3. Phytochemistry and Biological Activity. According to a comprehensive review of the chemical constituents of *P. sidoides* by Kolodziej [124], the extreme involvedness of the metabolites in *P. sidoides* is reflected by the occurrence of abundant coumarins, coumarin glycosides, coumarin sulphates, flavonoids, proanthocyanidins, phenolic acids, and phenylpropanoid derivatives. The antibacterial and antiviral properties are accredited to gallic acids and other phenolic compounds, while the immunomodulatory activity is alleged to be due to a combination of phenolic compounds and the numerous coumarins [122]. A systematic review and meta-analysis regarding the use of *P. sidoides* to treat acute bronchitis concluded that there is encouraging evidence from

currently available data indicating that *P. sidoides* is effective compared to placebo for patients with acute bronchitis [125]. Despite the commercial interest in *P. sidoides*, few studies have been conducted to document the phytochemical variation for natural populations [126].

8.4. Toxicity. A total of 18 clinical trials have thus far been conducted, several of which were randomised, double-blind, and placebo-controlled. Overall safety and a very low incidence of side effects were confirmed [122]. Recent reports ($n = 15$) of primarily assumed hepatotoxicity in connection with the use of *P. sidoides* have been interpreted by the Drug Commission of the German Medical Association (DCGMA). After analysis of these reports, Teschke et al. [127] concluded that there is little if any conclusive evidence and that assessments of these cases do not fulfil essential criteria required to propose the existence of *P. sidoides* hepatotoxicity.

8.5. Commercialization and Conservation. Since the 1990s, a prodelphinidin-rich ethanolic extract, made from the tuberous roots of *P. sidoides* called EPs 7630 (Umckaloabo), licensed to treat respiratory tract infections such as acute bronchitis, has become one of the most successful phytomedicines in the world [20, 128]. The annual sales in Germany alone exceed € 80 million [20]. A preparation of *P. sidoides* mother tincture is marketed in Ukraine, Russia, and Latvia as Umkalor [2]. Proprietary extracts of *P. sidoides* and their preparations as well as the use thereof are currently protected by seven patents worldwide [2]. Most of the plant material used is still being wild harvested by locals from rural communities in South Africa [129]. Official annual harvest data are not available but are estimated to be between 9,00 kg and 45,000 kg [2].

9. *Sclerocarya birrea* (Anacardiaceae)

9.1. Introduction. The name *Sclerocarya* is derived from the Greek word for hard (*skleros*) and nut (*karyon*) [2]. *Sclerocarya birrea* (A.Rich.) Hochst. subsp. caffra, also known as marula, is widely distributed in northern and western parts of Africa, but only the subspecies *caffra* is found in southern Africa [10]. Owing to its ability to provide two fundamental needs, namely, food and medicine [130], *S. birrea* is also referred to as the "tree of life" and is one of the most important trees of the southern African region. It is regarded sacred and protected in communal lands under the local chiefs [2]. Marula is a medium-sized deciduous tree of up to 15 meters in height with the male and female flowers habitually appearing on separate trees. The flowers are small, with red sepals and yellow petals [4]. The fruit, which averages around 20–30 g, is yellow when ripe with a white juicy flesh which clings to a hard stone, inside which are 2-3 seeds [2]. It is considered a drought-tolerant medicinal plant species with potential role in land rehabilitation [131].

9.2. Traditional Uses. Various cultures have utilized different plants of *S. birrea* to manage, control, and/or treat numerous ailments. For example, the bark is used to treat dysentery and diarrhea, rheumatism, and insect bites [2, 15] and is believed to be a cure for malaria and proctitis [38]. Essence from the leaves is said to provide relief from abscesses, burns and spider bites, whilst the marula oil is used as a balm to treat ear, nose, and throat conditions [2]. The fruit is used for the destruction of ticks [38]. It is interesting to note that leaves are predominantly used in Namibia where it is believed that the medicinal powers of male trees are greater than those of female trees, whereas the marula bark is more important in South Africa [132].

9.3. Phytochemistry and Biological Activity. The fruits of *S. birrea* are rich in ascorbic acid (168 mg/100 g) [133], and the fruit juice contains sesquiterpene hydrocarbons including caryophyllene, α-humulene, and copaene [134]. The fruit kernels contain high amounts of oil (47 mg/g dry weight mostly due to oleic acid), protein (36.4% dry weight), and are a good source of minerals (Cu, Mg, and Zn at 24.8, 4210, and 62.4 μg/g dry weight, resp.) [135]. The oil-rich seeds contain oleic, myristic, and stearic fatty acids and different types of amino acids, with a predominance of glutamic acid and arginine. However, when compared with the World Health Organization protein standard, the seeds contain low proportions of several of the essential amino acids, including leucine, lysine, the phenylalanine/tyrosine pair, and threonine [134]. The bark yields 5.5%–20.5% tannin and 10.7% tannin matter [15]. Numerous *in vitro* and *in vivo* studies to confirm biological activity include antidiarrheal, anti-inflammatory, antimalarial, antimicrobial, antiplasmodial, antihypertensive, anticonvulsant, antinociceptive, and antioxidant investigations which have been undertaken to verify traditional use of various plant parts [136–139]. It is suggested that *S. Birrea* stem bark extracts exert reno- and cardio-protective effects in diabetes mellitus [140]. Bark and leaf ethanolic extracts tested for antimicrobial activity ranged from 0.15 to 3 mg/mL [136]. Based on minimum inhibitory concentration values, inner bark extracts tended to be the most potent followed by outer bark and then the leaf extracts. Marula stem bark is shown to have exhibited high *in vitro* and *in vivo* (mouse model) antimalarial properties [138].

9.4. Toxicity. *In vitro* and *in vivo* toxicity studies of various *S. birrea* plant parts have shown conflicting results [141, 142]. A recent study on the effect of marula juice on albino rats has indicated that the marula juice may have an effect on liver and kidney functions at high dose [143].

9.5. Commercialization and Conservation. All parts of the marula tree can be used, making it one of the most valuable cultural and economic resources in southern Africa [144]. Its potential to generate cash income makes it a principally significant tree species which deserves more consideration in terms of utilization [130]. Marula has acquired significant commercial prominence since its fruits and other products entered local, regional, and international trade in the Southern Africa region; however, there are no reports on

commercialization of the species in East and Central regions of Africa [135]. In 1985, it was reported that 600 tonnes of juice was produced in South Africa alone [145]. Numerous small enterprises in southern Africa produce Marula jam and jellies [145].

The only product which has realised its full export potential is Amarula Cream. A marula cosmetic extract has been patented by Phytotrade in conjunction with Aldivia, a French company [2]. There is a threat that commercialisation of *S. birrea* will lead to the increased "privatisation" and the marginalization of certain groups from due benefits, or that commercialization will draw resources into trade and away from important subsistence uses [146].

10. *Siphonochilus aethiopicus* (Zingiberaceae)

10.1. Introduction. *Siphonochilus aethiopicus* (Schweinf.) B. L. Burtt is commonly known as wild ginger or African ginger, with a restricted distribution in South Africa where it is only found in Mpumalanga and the Northern Province and has become extinct in KwaZulu-Natal [10]. It also grows in Zimbabwe, Malawi, and Zambia. Wild ginger is regarded as one of the most important medicinal plant species of South Africa [15]. The rhizomes and roots are used for a variety of ailments, including coughs, colds, asthma, headache, candida, malaria, and influenza, but also for hysteria, pain and several other traditional and cultural practices [10, 15, 38, 147]. Wild ginger is the main commercial species that is relatively known as a result of its popularity in traditional medicine and concerns about its conservation status [147].

10.2. Traditional Uses. Wild ginger is one of the most important medicinal species with a long history of traditional use and one of the most popular of all traditional medicinal plants of southern Africa [147]. Wild ginger in South Africa is regarded as one of the most traded species and consequently has become overexploited and is now rare or extinct in the wild [148]. The increasing demand for this plant is leading to serious overharvesting from the wild and presents an opportunity for potentially profitable cultivation. The rhizomes are chewed fresh to treat asthma, hysteria, cold, flu, coughs, pain relief, dysmenorrhea, influenza, hysteria, as well as to treat malaria and also chewed by women during menstruation [10]. The highly aromatic roots have been reported to be used by Zulu people as a protection against lightning [10].

10.3. Phytochemistry and Biological Activity. Since *S. aethiopicus* is one of the most important medicinal plants in South Africa, much attention has been paid to the chemistry and biological activity of the plant. Studies revealed the healing properties of the rhizomes and roots for a variety of ailments including coughs, colds, asthma, headache, candida, and malaria [4, 15]. More recently, the application of the sesquiterpenoid siphonochilone and extracts containing siphonochilone from *S. aethiopicus* have been patented for application of the anti-inflammatory activities in formulations treating asthma and allergic conditions [9]. Two new furanoterpenoid derivatives, namely, 4aaH-3,5a,8ab-trimethyl-4,4a,9-tetrahydro-naphtho[2,3-b]-furan-8-one and 2-hydroxy-4aaH-3,5a,8ab-trimethyl-4,4a,9-tetrahydronaphtho[2,3-b]-furan-8-one, were also isolated from *S. aethiopicus* [10, 149, 150]. It is widely used in the treatment of pain and inflammation [114]. The composition of the essential oil was described by Viljoen et al. [150].

10.4. Toxicity. *Siphonochilus aethiopicus* was found to cause DNA damage as detected in the comet assay which raised some concern for the use of the plant [151]. It was expressed by Fennell et al. [114] that prescription of this plant for the treatment of ailments should be treated with caution, and rigorous toxicological and clinical studies are necessary before they are widely prescribed for use in traditional medicine as they can cause damage to the DNA and might therefore have long-term effects on the body.

10.5. Commercialisation and Conservation. In 1993, African ginger was identified as one of the South African species with commercial production potential as it is in high demand and short supply [8]. It is often quoted as being locally extinct in some parts of South Africa as a result of overexploitation [117, 148]. As more plants are harvested and overexploited for financial gain, the need for genetic conservation of medicinal plants is emphasized as more of the wild populations are facing extinction [152]. In a study by Mander [153], it was estimated that 1.9 tonnes of African ginger totalling a number of 52,000 plants is traded annually in KwaZulu-Natal. The current situation necessitates an alternative supply of medicinal plant material to meet demand. The only option for many species is cultivation at a large scale so that the wild species are maintained and become financial viable [8]. *S. aethiopicus* is easy to propagate and cultivate and successfully cultivated in the warm parts of South Africa [147]. Vegetative propagation is the preferred method as this is very efficient and also the fact that the seeds develop and mature underground and therefore are difficult to find and use for propagation [154]. Micropropagation has been established although not widely used and still not incorporated on large scale for production of wild ginger [4, 25]. Cultivation of wild ginger should be a financially viable operation in South Africa since there is always a demand, and the income generated justifies the effort. Street traders obtain around R140/kg while shops and healers obtain a price of up to R450/kg [153].

11. *Sutherlandia frutescens* (Fabaceae)

11.1. Introduction. *Sutherlandia frutescens* (L.) R.Br. belongs to the family Fabaceae commonly known as the legume, pea, or bean family. With the genus being restricted to southern Africa [10], this soft-wooded shrub with reedy stems is found in Botswana, Namibia, and South Africa [25]. The large red flowers, around 3 cm long, are followed shortly by bladder-like fruits [25]. *Sutherlandia frutescens* is commonly known as the cancer bush because of the reported use by Khoi-San and Cape Dutch people against internal cancers since 1895 [155, 156]. However, because of its adaptability, *S. frutescens*

has several other vernacular names referring to its ethnomedicinal significance. These include "*petola*" (Setswana) which means "it changes," implying that the plant changes the course of many illnesses towards a positive outcome and "*lerumo-lamadi*" (Northern Sesotho) meaning "the spear for the blood," indicating that Sutherlandia is a powerful blood-purifier and all-purpose tonic [157]. *Sutherlandia* along with *H. hemerocallidea* are the two main African medicinal plants used for treatment of HIV/AIDS and are endorsed by the South African Ministry of Health for HIV management [87].

11.2. Traditional Uses. *Sutherlandia frutescens* has a lengthy history of medicinal use in southern Africa. Mainly the leaves are used, but all above ground parts are often included [10]. It has been used in the traditional medicine systems of different cultural groups for a wide diversity of ailments, including stomach ailments, backache, diabetes, stress, fever, and wounds [40, 155]. It is used for internal cancers, but despite numerous claims and anecdotes, there is no scientific evidence to confirm this [10]. The dried leaves and other preparations are used as a general tonic [25].

11.3. Phytochemistry and Biological Activity. The chief constituents of *S. frutescens* alleged to be active include L-canavanine, GABA, and D-pinitol. L-canavanine is a nonprotein amino acid which is the L-2-amino-4-guanidinooxy structural analogue of L-arginine [87]. GABA and methionine are two amino acids which are highly prone to degradation through hydrolysis; thus, during production this needs to be taken into account [157]. A study by Mncwangi and Viljoen [157] investigating *S. frutescens* samples collected from different natural populations ($n = 87$) and from a commercial plantation ($n = 60$) revealed tremendous variation in the yields of the different compounds both within and between populations. For example, L-canavanine ranged from 0.14 to 13.58 mg/g. Amino acids collectively constituted between 10% and 15% (w/w) of dried *S. frutescens*. Proline, L-asparagine, and alanine were the most abundant amino acids and collectively represent almost 60% of total amino acid content; however, not all samples contained all the amino acids. The content of L-canavanine, arginine, GABA, and D-pinitol in seeds, field leaves, and *in vitro* leaves of *S. frutescens* was quantified by Shaik et al. [158]. The results revealed that the secondary metabolites significantly differed depending on the source. *In vitro* leaves had higher quantities of all compounds, except for D-pinitol, and the study highlighted the use of *in vitro* biology for the extraction of *S. frutescens* compounds.

11.4. Toxicity. The long history of traditional use, with no reports of any serious side effects, suggests that *Sutherlandia* can be considered as being generally safe [155]. The South African Ministry of Health has concluded that this product is safe based on primate safety studies [87]. Recent preclinical studies indicate that concomitant administration of *Sutherlandia* with prescription drugs (CYP3A4 substrates) could possibly lead to therapeutic failure and clinically relevant drug-herb interactions [159].

11.5. Commercialization and Conservation. In recent years, there has been a marked increase in both scientific and commercial interests in *Sutherlandia* [155]. The first small-scale cultivation and commercialisation of *Sutherlandia* started in the Cape Province more than 20 years ago [156]. The first branded products were tablets made from powdered leaves of a selected chemotype named SU1 type, the protypes and products of which have been marketed by Phyto Nova (Pty) Ltd since 2000 [20]. Phyto Nova now pursues large-scale cultivation of this medicinal plant [156]. Although it is not officially sanctioned by regulatory agencies in Europe and US, *Sutherlandia* is distributed worldwide through internet sources which claim it to be an immunity booster [159].

12. Conclusion

South Africa is home to thousands of plant species, each an industry for unique and useful chemical compounds. However, more pragmatic information on medicinal plants would enhance their value in agricultural landscapes by helping farmers improve their livelihoods and ensure environmental sustainability [130]. While it is anticipated that commercialization of certain medicinal plant species will bring financial remunerations to communities, this should not impair the subsistence sector, or sustainability of the resource itself [160]. A drawback of using nonrenewable parts such as bark as a source for the medicinal trade is that removal of bark can lead to the death of the plant. If bark can be traded for a high price, valuable plant populations may be collected to extinction in nature [136]. Traditional medicine has been used for treating diseases over centuries, and scientific studies have revealed that many of them are potentially toxic. The use of more polar solvents could be the reason for plant extracts being less toxic as the toxic compounds will be only extracted in more apolar solvents [115]. Drug-drug interactions are well-known phenomena, and the risks have long been realised. For example, interactions involving the concurrent use of sildenafil, a drug used for erectile dysfunction with nitrates and nitrites, can produce profound hypotension leading to decreased coronary perfusion and myocardial infarction. This potentially fatal drug interaction also led to the withdrawal of several sildenafil-containing herbal medications from the market [161]. The use of herbs may mimic, magnify, or oppose the effect of drugs and should therefore be used with care, and proper consultation and information should be provided by health care practitioners when mixing herbs and pharmaceutical drugs as interaction with most drugs is not known. Some examples of already identified herb-drug interactions include bleeding when warfarin is combined with ginkgo (*Ginkgo biloba*), mild serotonin syndrome in patients who mix St John's wort (*Hypericum perforatum*) with serotonin-reuptake inhibitors, and potentiation of oral and topical corticosteroids by liquorice (*Glycyrrhiza glabra*) and soluble fibres (including guar gum and psyllium) which can decrease the absorption of drugs. An example of South African herb-drug interactions is the use of devil's claw with the use of warfarin resulting in puprura [162]. In addition, *H. hemerocallidea* and

S. frutescens showed a negative interaction with antiretroviral medication, thus patients may be at risk from treatment failure, viral resistance, or drug toxicity [163].

Intellectual property rights (IPRs) can both positively and negatively impact the interests of primary producers. In order to appreciate benefits from the use of IPRs, communities need substantial financial and technical support [146]. Bioprospecting and regulation thereof has been heavily debated in the past years to minimize exploitation of South African resources without benefit and recognition for knowledge holders. This regulation has been observed by the scientific community as another hurdle in promoting and developing bioactives [164]. A more contemporary avenue for bioactives is the use of plants as vehicles to make recombinant proteins and other molecules for use as therapeutics. However, this is not well explored in Africa probably because few research laboratories are equipped to do this work [6].

The paper addressed just a few applications of the chemical components of the identified plants and the use as food, beverages, various medicinal uses, cosmetic industry, flavor, and fragrance industry to name a few. Some well-known plants and applications such as rooibos, marula, and buchu oil are already contributing to local communities' upliftment by creating enterprises both from wild harvesting and by cultivation of material. Others such as wild ginger, cancer bush, and the African potato still have potential to become international pharmaceutical and herbal products, and the full potential has not yet been exploited.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

The South African Medical Research Council, National Research Foundation (South Africa), the University of South Africa, and the University of KwaZulu-Natal are thanked for their financial assistance.

References

[1] C. Veilleux and S. R. King, *An Introduction to Ethnobotany*, 1996, http://www.accessexcellence.org/.

[2] A. Gurib-Fakim, T. Brendler, L. D. Philips, and J. N. Eloff, *Green Gold Success Stories Using Southern African Medicinal Plant Species*, AAMPS Publishing, 2010.

[3] C. A. M. Louw, T. J. C. Regnier, and L. Korsten, "Medicinal bulbous plants of South Africa and their traditional relevance in the control of infectious diseases," *Journal of Ethnopharmacology*, vol. 82, no. 2-3, pp. 147–154, 2002.

[4] B. E. Van wyk and N. Gericke, *People's Plants. A Guide to Useful Plants of Southern Africa*, Briza Publications, 2000.

[5] C. Geldenhuys and D. Mitchell, "Sustainable harvesting technologies," in *Commercialising Medicinal Plants-a Southern African Guide*, N. Diederichs, Ed., pp. 21–40, Sun Press, Stellenbosch, South Africa, 2006.

[6] E. P. Rybicki, R. Chikwamba, M. Koch, J. I. Rhodes, and J. H. Groenewald, "Plant-made therapeutics: an emerging platform in South Africa," *Biotechnology Advances*, vol. 30, no. 2, pp. 449–459, 2012.

[7] K. F. Wiersum, A. P. Dold, M. Husselman, and M. Cocks, "Cultivation of medicinal plants as a tool for biodiversity conservation and poverty alleviation in the Amatola region, South Africa," in *Medicinal and Aromatic Plants*, R. J. Bogers, L. E. Craker, and D. Lange, Eds., pp. 43–57, Springer, 2006.

[8] A. B. Cunningham, "African medicinal plants: setting priorities at the interface between conservation and primary health care," in *People and Plants Working Paper*, Unesco, 1993.

[9] N. Gericke, "Muthi to medicine," *South African Journal of Botany*, vol. 77, no. 4, pp. 850–856, 2011.

[10] B. van Wyk, B. van Oudsthoorn, and N. Gericke, *Medicinal Plants of South Africa*, Briza publications, 1997.

[11] P. Golblatt and J. C. Manning, *Cape Plants: a Conspectus of the Cape Flora of South Africa*, National Botanical Institute of South Africa, Pretoria, South Africa, 2000.

[12] A. Moolla, S. F. Van Vuuren, R. L. Van Zyl, and A. M. Viljoen, "Biological activity and toxicity profile of 17 Agathosma (Rutaceae) species," *South African Journal of Botany*, vol. 73, no. 4, pp. 588–592, 2007.

[13] M. Wichtl and N. G. Bisset, *Herbal Drugs and Phytopharmaceuticals*, CRC Press, Boca Raton, Fla, USA, 2000.

[14] M. Lis-Balchin, S. Hart, and E. Simpson, "Buchu (*Agathosma betulina* and *A. crenulata*, Rutaceae) essential oils: their pharmacological action on guinea-pig ileum and antimicrobial activity on microorganisms," *Journal of Pharmacy and Pharmacology*, vol. 53, no. 4, pp. 579–582, 2001.

[15] J. M. Watt and M. G. Breyer-Brandwijk, *The Medicinal and Poisonous Plants of Southern and Eastern Africa*, Churchill Livingstone, London, UK, 1962.

[16] D. Simpson, "Buchu—South Africa's amazing herbal remedy," *Scottish Medical Journal*, vol. 43, no. 6, pp. 189–191, 1998.

[17] A. A. J. Fluck, W. M. Mitchell, and H. M. Perry, "Comparison of buchu leaf oil," *Journal of the Science and Food Agriculture*, vol. 12, pp. 290–292, 1961.

[18] R. Kaiser, D. Lamparsky, and P. Schudel, "Analysis of buchu leaf oil," *Journal of Agricultural and Food Chemistry*, vol. 23, no. 5, pp. 943–950, 1975.

[19] M. A. Posthumus, T. A. Van Beek, N. F. Collins, and E. H. Graven, "Chemical composition of the essential oils of *Agathosma betulina*, *A. crenulata* and an *A. betulina* x crenulata hybrid (Buchu)," *Journal of Essential Oil Research*, vol. 8, no. 3, pp. 223–228, 1996.

[20] B. E. Van Wyk, "The potential of South African plants in the development of new medicinal products," *South African Journal of Botany*, vol. 77, no. 4, pp. 812–829, 2011.

[21] M. Sandasi, G. P. P. Kamatou, M. Baranska, and A. M. Viljoen, "Application of vibrational spectroscopy in the quality assessment of Buchu oil obtained from two commercially important Agathosma species (Rutaceae)," *South African Journal of Botany*, vol. 76, no. 4, pp. 692–700, 2010.

[22] A. Moolla and A. M. Viljoen, "'Buchu'—*Agathosma betulina* and *Agathosma crenulata* (Rutaceae): a review," *Journal of Ethnopharmacology*, vol. 119, no. 3, pp. 413–419, 2008.

[23] A. Lubbe and R. Verpoorte, "Cultivation of medicinal and aromatic plants for specialty industrial materials," *Industrial Crops and Products*, vol. 34, no. 1, pp. 785–801, 2011.

[24] W. Chen, B. van Wyk, I. Vermaak, and A. Viljoen, "Cape Aloes—A review of the phytochemistry, pharmacology and commercialisation of *Aloe ferox*," *Phytochemistry Letters*, vol. 2, pp. 1–12, 2012.

[25] N. Crouch, R. Symmonds, W. Spring, and N. Diederichs, "Facts sheets for growing popular medicinal plant species," in *Commercialising Medicinal Plants-A Southern African Guide*, N. Diederichs, Ed., pp. 97–142, Sun Press, Stellenbosch, South Africa, 2006.

[26] B. van Wyk and G. Smith, *Guide to the Aloes of South Africa*, Briza Publications, 1996.

[27] J. Pujol, *Naturaafrica—The Herbalist Handbook*, Jean Pujol natural healers foundation, Durban, South African, 1990.

[28] B. van Wyk, B. van Oudsthoorn, and N. Gericke, *Medicinal Plants of South African*, 2009.

[29] M. L. Magwa, M. Gundidza, R. M. Coopoosamy, and B. Mayekiso, "Chemical composition of volatile constituents from the leaves of *Aloe ferox*," *African Journal of Biotechnology*, vol. 5, no. 18, pp. 1652–1654, 2006.

[30] O. A. Wintola, T. O. Sunmonu, and A. J. Afolayan, "The effect of *Aloe ferox* Mill. In the treatment of loperamide-induced constipation in Wistar rats," *BMC Gastroenterology*, vol. 10, article 95, 2010.

[31] D. T. Loots, F. H. Van Der Westhuizen, and L. Botes, "*Aloe ferox* leaf gel phytochemical content, antioxidant capacity, and possible health benefits," *Journal of Agricultural and Food Chemistry*, vol. 55, no. 17, pp. 6891–6896, 2007.

[32] M. Mwale and P. J. Masika, "Analgesic and anti-inflammatory activities of *Aloe ferox* Mill. aqueous extract," *African Journal of Pharmacy and Pharmacology*, vol. 4, no. 6, pp. 291–297, 2010.

[33] O. A. Fawole, S. O. Amoo, A. R. Ndhlala, M. E. Light, J. F. Finnie, and J. Van Staden, "Anti-inflammatory, anticholinesterase, antioxidant and phytochemical properties of medicinal plants used for pain-related ailments in South Africa," *Journal of Ethnopharmacology*, vol. 127, no. 2, pp. 235–241, 2010.

[34] L. Kambizi, N. Sultana, and A. J. Afolayan, "Bioactive compounds isolated from *Aloe ferox*: a plant traditionally used for the treatment of sexually transmitted infections in the Eastern Cape, South Africa," *Pharmaceutical Biology*, vol. 42, no. 8, pp. 636–639, 2005.

[35] V. Maphosa, P. J. Masika, E. S. Bizimenyera, and J. N. Eloff, "In-vitro anthelminthic activity of crude aqueous extracts of *Aloe ferox*, *Leonotis leonurus* and *Elephantorrhiza elephantina* against *Haemonchus contortus*," *Tropical Animal Health and Production*, vol. 42, no. 2, pp. 301–307, 2010.

[36] S. Kametani, A. Kojima-Yuasa, H. Kikuzaki, D. O. Kennedy, M. Honzawa, and I. Matsui-Yuasa, "Chemical constituents of cape *Aloe* and their synergistic growth-inhibiting effect on ehrlich ascites tumor cells," *Bioscience, Biotechnology and Biochemistry*, vol. 71, no. 5, pp. 1220–1229, 2007.

[37] D. T. Loots, M. Pieters, M. S. Islam, and L. Botes, "Antidiabetic effects of *Aloe ferox* and *Aloe greatheadii* var. Davyana leaf gel extracts in a low-dose streptozotocin diabetes rat model," *South African Journal of Science*, vol. 107, no. 7-8, 2011.

[38] A. Hutchings, A. H. Scott, G. Lewis, and A. Cunningham, *Zulu Medicinal Plants—An Inventory*, University of Natal Press Pietermaritzburg, Pietermaritzburg, South Africa, 1996.

[39] B. E. Van Wijk, M. C. B. R. Van Oudtshoorn, and G. F. Smith, "Geographical variation in the major compounds of *Aloe ferox* leaf exudate," *Planta Medica*, vol. 61, no. 3, pp. 250–253, 1995.

[40] S. E. Drewes, M. Horn, and F. Khan, "The chemistry and pharmacology of medicinal plants," in *Commercialising Medicinal Plants—A Southern African Guide*, N. Diederichs, Ed., pp. 87–96, Sun Press, Stellenbosch, South Africa, 2006.

[41] D. J. Newton and H. Vaughan, *South Africa's Aloe ferox Plant Parts and Derivatives Industry*, TRAFFIC East/Southern Africa, Johannesburg, South Africa, 1996.

[42] C. M. Shackleton and J. Gambiza, "Growth of *Aloe ferox* Mill. at selected sites in the Makana region of the Eastern Cape," *South African Journal of Botany*, vol. 73, no. 2, pp. 266–269, 2007.

[43] O. M. Grace, "Current perspectives on the economic botany of the genus *Aloe L.*, (Xanthorrhoeaceae)," *South African Journal of Botany*, vol. 77, no. 4, pp. 980–987, 2011.

[44] J. Gruenwald, "Novel botanical ingredients for beverages," *Clinics in Dermatology*, vol. 27, no. 2, pp. 210–216, 2009.

[45] E. Joubert and D. de Beer, "Rooibos (*Aspalathus linearis*) beyond the farm gate: from herbal tea to potential phytopharmaceutical," *South African Journal of Botany*, vol. 77, no. 4, pp. 869–886, 2011.

[46] E. Joubert, W. C. A. Gelderblom, A. Louw, and D. de Beer, "South African herbal teas: *Aspalathus linearis*, *Cyclopia* spp. and *Athrixia phylicoides*—a review," *Journal of Ethnopharmacology*, vol. 119, no. 3, pp. 376–412, 2008.

[47] I. S. Koch, M. Muller, E. Joubert, M. van der Rijst, and T. Næs, "Sensory characterization of rooibos tea and the development of a rooibos sensory wheel and lexicon," *Food Research International*, vol. 46, no. 1, pp. 217–228, 2012.

[48] D. A. Mulholland and S. E. Drewes, "Global phytochemistry: indigenous medicinal chemistry on track in southern Africa," *Phytochemistry*, vol. 65, no. 7, pp. 769–782, 2004.

[49] K. L. J. Blommaert and J. Steenkamp, "Tannin and possible caffeine content of rooibos tea, *Aspalathus* (Subgen. Nortiera) *linearis* (Burm. Fil) R. Dahlgr [Afrikaans]," *Agroplantae*, vol. 10, p. 49, 1978.

[50] B.-E. Van Wyk and G. H. Verdoorn, "Alkaloids of the genera *Aspalathus*, Rafnia and Wiborgia (Fabaceae-Crotalarieae)," *South African Journal of Botany*, vol. 55, pp. 520–522, 1989.

[51] B. H. Koeppen and D. G. Roux, "Aspalathin: a novel C-glycosylflavonoid from *Aspalathus linearis*," *Tetrahedron Letters*, vol. 6, no. 39, pp. 3497–3503, 1965.

[52] N. Shimamura, T. Miyase, K. Umehara, T. Warashina, and S. Fujii, "Phytoestrogens from *Aspalathus linearis*," *Biological and Pharmaceutical Bulletin*, vol. 29, no. 6, pp. 1271–1274, 2006.

[53] P. W. Snijman, S. Swanevelder, E. Joubert, I. R. Green, and W. C. A. Gelderblom, "The antimutagenic activity of the major flavonoids of rooibos (*Aspalathus linearis*): some dose-response effects on mutagen activation-flavonoid interactions," *Mutation Research—Genetic Toxicology and Environmental Mutagenesis*, vol. 631, no. 2, pp. 111–123, 2007.

[54] K. Kunishiro, A. Tai, and I. Yamamoto, "Effects of Rooibos tea extract on antigen-specific antibody production and cytokine generation in vitro and in vivo," *Bioscience, Biotechnology and Biochemistry*, vol. 65, no. 10, pp. 2137–2145, 2001.

[55] S. Scheepers, *Anti-microbial activity of rooibos tea (*Aspalathus linearis*) on food spoilage organisms and potential pathogens*, Stellenbosch University, 2001.

[56] J. L. Marnewick, F. Rautenbach, I. Venter et al., "Effects of rooibos (*Aspalathus linearis*) on oxidative stress and biochemical parameters in adults at risk for cardiovascular disease," *Journal of Ethnopharmacology*, vol. 133, no. 1, pp. 46–52, 2011.

[57] D. Ferreira, C. Marais, J. A. Steenkamp, and E. Joubert, "Rooibos tea as a likely health food supplements," in *Proceedings in Recent Development of Technologies on Fundamental Foods for Health*, 1995.

[58] B. H. Koeppen, J. B. Smit, and D. G. Roux, "The flavone *C*-glycosides and flavonol *O*-glycosides of *Aspalathus acuminatus* (rooibos tea)," *The Biochemical Journal*, vol. 83, pp. 507–511, 1962.

[59] C. Marais, W. J. Van Rensburg, D. Ferreira, and J. A. Steenkamp, "(S)- and (R)-eriodictyol-6-C-β-D-glucopyranoside, novel keys to the fermentation of rooibos (*Aspalathus linearis*)," *Phytochemistry*, vol. 55, no. 1, pp. 43–49, 2000.

[60] C. Rabe, J. A. Steenkamp, E. Joubert, J. F. W. Burger, and D. Ferreira, "Phenolic metabolites from rooibos tea (*Aspalathus linearis*)," *Phytochemistry*, vol. 35, no. 6, pp. 1559–1565, 1994.

[61] R. Beltrán-Debón, A. Rull, F. Rodríguez-Sanabria et al., "Continuous administration of polyphenols from aqueous rooibos (*Aspalathus linearis*) extract ameliorates dietary-induced metabolic disturbances in hyperlipidemic mice," *Phytomedicine*, vol. 18, no. 5, pp. 414–424, 2011.

[62] T. Breiter, C. Laue, G. Kressel, S. Gröll, U. H. Engelhardt, and A. Hahn, "Bioavailability and antioxidant potential of rooibos flavonoids in humans following the consumption of different rooibos formulations," *Food Chemistry*, vol. 128, no. 2, pp. 338–347, 2011.

[63] N. Krafczyk and M. A. Glomb, "Characterization of phenolic compounds in rooibos tea," *Journal of Agricultural and Food Chemistry*, vol. 56, no. 9, pp. 3368–3376, 2008.

[64] C. Marais, J. A. Steenkamp, and D. Ferreira, "The occurrence of phenylpyruvic acid in woody plants: biosynthetic significance," *Tetrahedron Letters*, vol. 37, no. 32, pp. 5763–5764, 1996.

[65] J. F. Morton, "Rooibos tea, *Aspalathus linearis*, a caffeineless, low-tannin beverage," *Economic Botany*, vol. 37, pp. 164–173, 1983.

[66] E. Nel, T. Binns, and D. Bek, "'Alternative foods' and community-based development: rooibos tea production in South Africa's West Coast Mountains," *Applied Geography*, vol. 27, no. 2, pp. 112–129, 2007.

[67] S. Patrickson, R. Malgas, and N. Oettlé, "Environmental threat or conservation opportunity?" *Veld & Flora*, vol. 94, pp. 8–11, 2008.

[68] T. Binns, D. Bek, E. Nel, and B. Ellison, "Sidestepping the mainstream: fairtrade rooibos tea production in Wupperthal, South Africa," in *Alternative Food Geographies*, D. Maye, L. Holloway, and M. Kneafsey, Eds., pp. 331–349, Elsevier, Amsterdam, The Netherlands, 2007.

[69] Anon, "Stock situation [Afrikaans]," *Die Rooibos*, vol. 2, p. 4, 1994.

[70] L. Grant, D. E. McBean, L. Fyfe, and A. M. Warnock, "A review of the biological and potential therapeutic actions of *Harpagophytum procumbens*," *Phytotherapy Research*, vol. 21, no. 3, pp. 199–209, 2007.

[71] N. Mncwangi, W. Chen, I. Vermaak, A. M. Viljoen, and N. Gericke, "Devil's claw-a review of the ethnobotany, phytochemistry and biological activity of *Harpagophytum procumbens*," *Journal of Ethnopharmacology*, vol. 143, no. 3, pp. 755–771, 2012.

[72] J. Barnes, "Devil's claw (*Harpagophytum procumbens*). Also known as "grapple plant" or "wood spider"," *Journal of Primary Health Care*, vol. 1, no. 3, pp. 238–239, 2009.

[73] V. Gyurkovska, K. Alipieva, A. Maciuk et al., "Anti-inflammatory activity of Devil's claw in vitro systems and their active constituents," *Food Chemistry*, vol. 125, no. 1, pp. 171–178, 2011.

[74] J. Qi, N. Li, J. H. Zhou, B. Y. Yu, and S. X. Qiu, "Isolation and anti-inflammatory activity evaluation of triterpenoids and a monoterpenoid glycoside from *Harpagophytum procumbens*," *Planta Medica*, vol. 76, no. 16, pp. 1892–1896, 2010.

[75] B. L. Fiebich, E. Muñoz, T. Rose, G. Weiss, and G. P. McGregor, "Molecular targets of the antiinflammatory *Harpagophytum procumbens* (Devil's claw): inhibition of TNFα and COX-2 gene expression by preventing activation of AP-1," *Phytotherapy Research*, vol. 26, no. 6, pp. 806–811, 2012.

[76] L. Zhang, L. Feng, Q. Jia et al., "Effects of β-glucosidase hydrolyzed products of harpagide and harpagoside on cyclooxygenase-2 (COX-2) in vitro," *Bioorganic and Medicinal Chemistry*, vol. 19, no. 16, pp. 4882–4886, 2011.

[77] J. Vlachojannis, B. D. Roufogalis, S. Chrubasik, and J. Chrubasik, "Systematic review on the safety of Harpagophytum preparations for osteoarthritic and low back pain," *Phytotherapy Research*, vol. 22, no. 2, pp. 149–152, 2008.

[78] K. M. Stewart and D. Cole, "The commercial harvest of devil's claw (*Harpagophytum* spp.) in southern Africa: the devil's in the details," *Journal of Ethnopharmacology*, vol. 100, no. 3, pp. 225–236, 2005.

[79] D. Raimondo and J. Donaldson, "The trade, management and biological status of *Harpagophytum* spp. in southern African range states. and L. T. N. A report submitted to the twelfth meeting of the CITES Plants Committee," in *A report submitted to the twelfth meeting of the CITES Plants Committee*, The trade, management and biological status of Harpagophytum spp. in southern African range states, Leiden, The Netherlands, May 2002.

[80] S. E. Drewes, A. J. Hall, R. A. Learmonth, and U. J. Upfold, "Isolation of hypoxoside from *Hypoxis rooperi* and synthesis of (E)-1,5-bis(3′,4′-dimethoxyphenyl)pent-4-en-1-yne," *Phytochemistry*, vol. 23, no. 6, pp. 1313–1316, 1984.

[81] Y. Singh, "Hypoxis: Yellow stars of horticulture, folk remedies and conventional medicine," *Veld and Flora*, vol. 85, pp. 123–125, 1999.

[82] S. E. Drewes, E. Elliot, F. Khan, J. T. B. Dhlamini, and M. S. S. Gcumisa, "*Hypoxis hemerocallidea*—Not merely a cure for benign prostate hyperplasia," *Journal of Ethnopharmacology*, vol. 119, no. 3, pp. 593–598, 2008.

[83] Y. Singh, "Hypoxis (Hypoxidaceae) in southern Africa: taxonomic notes," *South African Journal of Botany*, vol. 73, no. 3, pp. 360–365, 2007.

[84] O. Laporta, L. Pérez-Fons, R. Mallavia, N. Caturla, and V. Micol, "Isolation, characterization and antioxidant capacity assessment of the bioactive compounds derived from *Hypoxis rooperi* corm extract (African potato)," *Food Chemistry*, vol. 101, no. 4, pp. 1425–1437, 2007.

[85] J. A. O. Ojewole, "Antinociceptive, anti-inflammatory and antidiabetic properties of *Hypoxis hemerocallidea* Fisch. & C.A. Mey. (Hypoxidaceae) corm ['African Potato'] aqueous extract in mice and rats," *Journal of Ethnopharmacology*, vol. 103, no. 1, pp. 126–134, 2006.

[86] V. Steenkamp, M. C. Gouws, M. Gulumian, E. E. Elgorashi, and J. Van Staden, "Studies on antibacterial, anti-inflammatory and antioxidant activity of herbal remedies used in the treatment of benign prostatic hyperplasia and prostatitis," *Journal of Ethnopharmacology*, vol. 103, no. 1, pp. 71–75, 2006.

[87] E. Mills, C. Cooper, D. Seely, and I. Kanfer, "African herbal medicines in the treatment of HIV: hypoxis and Sutherlandia. An overview of evidence and pharmacology," *Nutrition Journal*, vol. 4, no. 19, pp. 1–8, 2005.

[88] O. O. G. Oluwule, N. A. Sukati, P. S. Dlamini, and F. G. Sibandze, "Some Swazi phytomedicines and their constituents," *African Journal of Biotechnology*, vol. 6, no. 3, pp. 267–272, 2007.

[89] V. Steenkamp, "Phytomedicines for the prostate," *Fitoterapia*, vol. 74, no. 6, pp. 545–552, 2003.

[90] C. T. Musabayane, K. Xozwa, and J. A. O. Ojewole, "Effects of *Hypoxis hemerocallidea* (Fisch. & C.A. Mey.) [Hypoxidaceae] corm (african potato) aqueous extract on renal electrolyte and fluid handling in the rat," *Renal Failure*, vol. 27, no. 6, pp. 763–770, 2005.

[91] C. F. Albrecht, P. B. Kruger, B. J. Smit et al., "The pharmacokinetic behaviour of hypoxoside taken orally by patients with lung cancer in a phase I trial," *South African Medical Journal*, vol. 85, no. 9, pp. 861–865, 1995.

[92] D. R. Katerere and J. N. Eloff, "Anti-bacterial and anti-oxidant activity of *Hypoxis hemerocallidea* (Hypoxidaceae): can leaves be substituted for corms as a conservation strategy?" *South African Journal of Botany*, vol. 74, no. 4, pp. 613–616, 2008.

[93] A. P. Dold and M. L. Cocks, "The trade in medicinal plants in the Eastern Cape Province, South Africa," *South African Journal of Science*, vol. 98, no. 11-12, pp. 589–597, 2002.

[94] R. D. Hammerton and J. Van Staden, "Seed germination of *Hypoxis hemerocallidea*," *South African Journal of Botany*, vol. 54, no. 3, pp. 277–280, 1988.

[95] D. A. Mulholland and S. E. Drewes, "Global phytochemistry: indigenous medicinal chemistry on track in southern Africa," *Phytochemistry*, vol. 65, no. 7, pp. 769–782, 2004.

[96] Y. M. Page and J. Van Staden, "In vitro propagation of *Hypoxis rooperi*," *Plant Cell, Tissue and Organ Culture*, vol. 3, no. 4, pp. 359–362, 1984.

[97] Y. M. Page and J. Van Staden, "In vitro propagation of *Hypoxis rooperi* from flower buds," *South African Journal of Botany*, vol. 52, no. 2, pp. 261–264, 1986.

[98] R. D. Hammerton, M. T. Smith, and J. Van Staden, "Factors influencing seed variability and germination in *Hypoxis hemerocallidea*," *Seed Science and Technology*, vol. 17, no. 3, pp. 613–624, 1989.

[99] Y. M. Page and J. Van Staden, "Hypoxoside production in tissue cultures of *Hypoxis rooperi*," *Plant Cell, Tissue and Organ Culture*, vol. 9, no. 2, pp. 131–136, 1987.

[100] S. G. Sparg, A. K. Jäger, and J. Van Staden, "The effect of cultivation and plant age on the pharmacological activity of *Merwilla natalensis* bulbs," *South African Journal of Botany*, vol. 71, no. 2, pp. 191–196, 2005.

[101] V. L. Williams, D. Raimondo, and A. B. Cunningham, "Trade, bulb age and impacts on *Merwilla plumbea*," *South African Journal of Botany*, vol. 73, no. 2, pp. 321–322, 2007.

[102] S. G. Sparg, A. K. Jäger, M. L. Magwa, and J. Van Staden, "Cultivating the medicinal plant *Merwilla natalensis* as a crop. A small-scale farming approach," *Outlook on Agriculture*, vol. 34, no. 2, pp. 116–120, 2005.

[103] S. G. Sparg, J. Van Staden, and A. K. Jäger, "Pharmacological and phytochemical screening of two Hyacinthaceae species: *Scilla natalensis* and *Ledebouria ovatifolia*," *Journal of Ethnopharmacology*, vol. 80, no. 1, pp. 95–101, 2002.

[104] N. Moodley, D. A. Mulholland, and N. R. Crouch, "Eucosterol-type nortriterpenoids from *Merwilla natalensis*," *Journal of Natural Products*, vol. 67, no. 5, pp. 918–920, 2004.

[105] A. Notten, Merwilla plumbea *(Lindl.) Speta*, 2001, http://www.plantzafrica.com.

[106] B. Ncube, J. F. Finnie, and J. Van Staden, "Seasonal variation in antimicrobial and phytochemical properties of frequently used medicinal bulbous plants from South Africa," *South African Journal of Botany*, vol. 77, no. 2, pp. 387–396, 2011.

[107] T. S. Kellermen, J. A. W. Coetzer, and T. W. Naude, *Plant Poisonings and Mycotoxicoses of Livestock in South Africa*, Oxford University Press, Cape Town, South Africa, 1988.

[108] K. Du Toit, E. E. Elgorashi, S. F. Malan et al., "Anti-inflammatory activity and QSAR studies of compounds isolated from Hyacinthaceae species and *Tachiadenus longiflorus* Griseb. (Gentianaceae)," *Bioorganic and Medicinal Chemistry*, vol. 13, no. 7, pp. 2561–2568, 2005.

[109] K. Du Toit, E. E. Elgorashi, S. F. Malan, D. A. Mulholland, S. E. Drewes, and J. Van Staden, "Antibacterial activity and QSAR of homoisoflavanones isolated from six Hyacinthaceae species," *South African Journal of Botany*, vol. 73, no. 2, pp. 236–241, 2007.

[110] N. R. Crouch, V. Bangani, and D. A. Mulholland, "Homoisoflavanones from three South African Scilla species," *Phytochemistry*, vol. 51, no. 7, pp. 943–946, 1999.

[111] B. S. Kirkiacharian, M. Gomis, H. G. Tongo, J. Mahuteau, and J. D. Brion, *Organic Magnetic Resonance*, John Wiley & sons, 1984.

[112] G. I. Stafford, A. K. Jäger, and J. Van Staden, "Effect of storage on the chemical composition and biological activity of several popular South African medicinal plants," *Journal of Ethnopharmacology*, vol. 97, no. 1, pp. 107–115, 2005.

[113] E. E. Elgorashi, J. L. S. Taylor, A. Maes, J. Van Staden, N. De Kimpe, and L. Verschaeve, "Screening of medicinal plants used in South African traditional medicine for genotoxic effects," *Toxicology Letters*, vol. 143, no. 2, pp. 195–207, 2003.

[114] C. W. Fennell, K. L. Lindsey, L. J. McGaw et al., "Assessing African medicinal plants for efficacy and safety: pharmacological screening and toxicology," *Journal of Ethnopharmacology*, vol. 94, no. 2-3, pp. 205–217, 2004.

[115] B. G. Ndawonde, A. M. Zobolo, E. T. Dlamini, and S. J. Siebert, "A survey of plants sold by traders at Zululand muthi markets, with a view to selecting popular plant species for propagation in communal gardens," *African Journal of Range and Forage Science*, vol. 24, no. 2, pp. 103–107, 2007.

[116] R. A. Street, M. G. Kulkarni, W. A. Stirk et al., "Effect of cadmium uptake and accumulation on growth and antibacterial activity of *Merwilla plumbea*—an extensively used medicinal plant in South Africa," *South African Journal of Botany*, vol. 75, no. 3, pp. 611–616, 2009.

[117] A. B. Cunningham, *An Investigation of the Herbal Medicinal Trade in Natal/KwaZulu*, Institute of Natural Resources, 1988.

[118] S. Zschocke, T. Rabe, J. L. S. Taylor, A. K. Jäger, and J. Van Staden, "Plant part substitution—a way to conserve endangered medicinal plants?" *Journal of Ethnopharmacology*, vol. 71, no. 1-2, pp. 281–292, 2000.

[119] S. G. Sparg, M. G. Kulkarni, and J. Van Staden, "Germination and seedling establishment strategies for *Merwilla natalensis*, a South African medicinal plant in high demand," *South African Journal of Science*, vol. 101, no. 3-4, pp. 205–208, 2005.

[120] H. Kolodziej, "Antimicrobial, antiviral and immunomodulatory activity studies of *Pelargonium sidoides* (EPs 7630) in the context of health promotion," *Pharmaceuticals*, vol. 4, pp. 1295–1314, 2011.

[121] E. Lawrence, *Pelargonium Sidoides DC*, 2001, http://www.plantzafrica.com/plantnop/pelargsidoid.htm.

[122] T. Brendler and B. E. van Wyk, "A historical, scientific and commercial perspective on the medicinal use of *Pelargonium sidoides* (Geraniaceae)," *Journal of Ethnopharmacology*, vol. 119, no. 3, pp. 420–433, 2008.

[123] B. Matsiliza and N. P. Barker, "A preliminary survey of plants used in traditional medicine in the Grahamstown area," *South African Journal of Botany*, vol. 67, no. 2, pp. 177–182, 2001.

[124] H. Kolodziej, "Fascinating metabolic pools of *Pelargonium sidoides* and *Pelargonium reniforme*, traditional and phytomedicinal sources of the herbal medicine Umckaloabo," *Phytomedicine*, vol. 14, no. 1, pp. 9–17, 2007.

[125] T. B. Agbabiaka, R. Guo, and E. Ernst, "*Pelargonium sidoides* for acute bronchitis: a systematic review and meta-analysis," *Phytomedicine*, vol. 15, no. 5, pp. 378–385, 2008.

[126] J. E. Maree and A. M. Viljoen, "Phytochemical distinction between *Pelargonium sidoides* and *Pelargonium reniforme*—a quality control perspective," *South African Journal of Botany*, vol. 82, no. 0, pp. 83–91, 2012.

[127] R. Teschke, C. Frenzel, J. Schulze, and A. Eickhoff, "Spontaneous reports of primarily suspected herbal hepatotoxicity by *Pelargonium sidoides*: was causality adequately ascertained?" *Regulatory Toxicology and Pharmacology*, vol. 63, no. 1, pp. 1–9, 2012.

[128] L. L. Theisen and C. P. Muller, "EPs 7630 (Umckaloabo), an extract from *Pelargonium sidoides* roots, exerts anti-influenza virus activity in vitro and in vivo," *Antiviral Research*, vol. 94, no. 2, pp. 147–156, 2012.

[129] F. B. Lewu, P. O. Adebola, and A. J. Afolayan, "Commercial harvesting of *Pelargonium sidoides* in the Eastern Cape, South Africa: striking a balance between resource conservation and rural livelihoods," *Journal of Arid Environments*, vol. 70, no. 2, pp. 380–388, 2007.

[130] G. N. Gouwakinnou, A. M. Lykke, A. E. Assogbadjo, and B. Sinsin, "Local knowledge, pattern and diversity of use of *Sclerocarya birrea*," *Journal of Ethnobiology and Ethnomedicine*, vol. 7, article 8, 2011.

[131] J. D. H. Lambert, P. A. Ryden, and E. E. Esikuri, *Capitalizing on the Bio-Economic Value of Multi-Purpose Medicinal Plants for the Rehabilitation of Drylands in Sub-Saharan Africa*, 2005.

[132] S. Shackleton, S. Den Adel, T. McHardy, and C. Shackleton, *Use of Marula Products for Domestic and Commercial Purposes: Synthesis of Key Findings from Three Sites in Southern Africa*, G.D.f.I. Development, 2002.

[133] A. M. Viljoen, G. P. P. Kamatou, and K. H. C. Başer, "Headspace volatiles of marula (*Sclerocarya birrea* subsp. *caffra*)," *South African Journal of Botany*, vol. 74, no. 2, pp. 325–326, 2008.

[134] A. A. Mariod and S. I. Abdelwahab, "*Sclerocarya birrea* (Marula), an African tree of nutritional and medicinal uses: a review," *Food Reviews International*, vol. 28, no. 4, pp. 375–388, 2012.

[135] B. A. Jama, A. M. Mohamed, J. Mulatya, and A. N. Njui, "Comparing the "Big Five": a framework for the sustainable management of indigenous fruit trees in the drylands of East and Central Africa," *Ecological Indicators*, vol. 8, no. 2, pp. 170–179, 2008.

[136] J. N. Eloff, "Antibacterial activity of Marula (*Sclerocarya birrea* (A. rich.) Hochst. subsp. *caffra* (Sond.) Kokwaro) (Anacardiaceae) bark and leaves," *Journal of Ethnopharmacology*, vol. 76, no. 3, pp. 305–308, 2001.

[137] J. A. O. Ojewole, T. Mawoza, W. D. H. Chiwororo, and P. M. O. Owira, "*Sclerocarya birrea* (A. Rich) Hochst. ["Marula"] (Anacardiaceae): a review of its phytochemistry, pharmacology and toxicology and its ethnomedicinal uses," *Phytotherapy Research*, vol. 24, no. 5, pp. 633–639, 2010.

[138] J. W. Gathirwa, G. M. Rukunga, E. N. M. Njagi et al., "The in vitro anti-plasmodial and in vivo anti-malarial efficacy of combinations of some medicinal plants used traditionally for treatment of malaria by the Meru community in Kenya," *Journal of Ethnopharmacology*, vol. 115, no. 2, pp. 223–231, 2008.

[139] M. Moyo, A. R. Ndhlala, J. F. Finnie, and J. Van Staden, "Phenolic composition, antioxidant and acetylcholinesterase inhibitory activities of *Sclerocarya birrea* and *Harpephyllum caffrum* (Anacardiaceae) extracts," *Food Chemistry*, vol. 123, no. 1, pp. 69–76, 2010.

[140] M. Gondwe, D. R. Kamadyaapa, M. Tufts, A. A. Chuturgoon, and C. T. Musabayane, "*Sclerocarya birrea* [(A. Rich.) Hochst.] [Anacardiaceae] stem-bark ethanolic extract (SBE) modulates blood glucose, glomerular filtration rate (GFR) and mean arterial blood pressure (MAP) of STZ-induced diabetic rats," *Phytomedicine*, vol. 15, no. 9, pp. 699–709, 2008.

[141] L. Verschaeve and J. Van Staden, "Mutagenic and antimutagenic properties of extracts from South African traditional medicinal plants," *Journal of Ethnopharmacology*, vol. 119, no. 3, pp. 575–587, 2008.

[142] M. van de Venter, S. Roux, L. C. Bungu et al., "Antidiabetic screening and scoring of 11 plants traditionally used in South Africa," *Journal of Ethnopharmacology*, vol. 119, no. 1, pp. 81–86, 2008.

[143] L. G. Hassan, S. M. Dangoggo, S. W. Hassan, S. Muhammad, and K. J. Umar, "Serum biochemical response of rats fed with *Sclerocarya birrea* juice extracts," *African Journal of Food Science*, vol. 5, no. 4, pp. 208–212, 2011.

[144] N. C. Mokgolodi, Y. F. Ding, M. P. Setshogo, C. Ma, and Y. J. Liu, "The importance of an indigenous tree to southern African communities with specific relevance to its domestication and commercialization: a case of the marula tree," *Forestry Studies in China*, vol. 13, no. 1, pp. 36–44, 2011.

[145] R. R. B. Leakey, "Potential for novel food products from agroforestry trees: a review," *Food Chemistry*, vol. 66, no. 1, pp. 1–14, 1999.

[146] R. P. Wynberg, S. A. Laird, S. Shackleton et al., "Marula policy brief: marula commercialisation for sustainable and equitable livelihoods," *Forests Trees and Livelihoods*, vol. 13, no. 3, pp. 203–215, 2003.

[147] B. E. van Wyk, "A broad review of commercially important southern African medicinal plants," *Journal of Ethnopharmacology*, vol. 119, no. 3, pp. 342–355, 2008.

[148] N. Diederichs, C. Geldenhuys, and D. Mitchell, *The First Legal Harvesters of Protected Medicinal Plants in South Africa*, 2002, http://www.scienceinafrica.co.za/2002/november/bark.htm.

[149] C. W. Holzapfel, W. Marais, P. L. Wessels, and B. E. Van Wyk, "Furanoterpenoids from *Siphonochilus aethiopicus*," *Phytochemistry*, vol. 59, no. 4, pp. 405–407, 2002.

[150] A. M. Viljoen, B. Demirci, K. H. C. Başer, and B. E. Van Wyk, "The essential oil composition of the roots and rhizomes of *Siphonochilus aethiopicus*," *South African Journal of Botany*, vol. 68, no. 1, pp. 115–116, 2002.

[151] J. L. S. Taylor, E. E. Elgorashi, A. Maes et al., "Investigating the safety of plants used in South African traditional medicine: testing for genotoxicity in the micronucleus and alkaline comet assays," *Environmental and Molecular Mutagenesis*, vol. 42, no. 3, pp. 144–154, 2003.

[152] V. Heywood, "Medicinal and aromatic plants as global resources," *Acta Horticulturae (ISHS)*, vol. 500, pp. 21–30, 1999.

[153] M. Mander, "Marketing of indigenous medicinal plants of South Africa: a case study in Kwazulu-Natal. Food and Agricultural Organization of the United Nations," *Food and Agricultural Organization of the United Nations*, 1998.

[154] N. R. Crouch, M. C. Lotter, S. Krynauw, and C. Pottas-Bircher, "*Siphonochilus aethiopicus* (Zingiberaceae), the Prized Indungulu of the Zulu-an overview," *Herbertia*, vol. 55, pp. 115–129, 2000.

[155] B. E. van Wyk and C. Albrecht, "A review of the taxonomy, ethnobotany, chemistry and pharmacology of *Sutherlandia frutescens* (Fabaceae)," *Journal of Ethnopharmacology*, vol. 119, no. 3, pp. 620–629, 2008.

[156] S. Drewes, "Natural products research in South Africa: 1890–2010," *South African Journal of Science*, vol. 108, no. 5/6, pp. 769–782, 2012.

[157] N. P. Mncwangi and A. M. Viljoen, "Quantitative variation of amino acids in *Sutherlandia frutescens* (Cancer bush)-towards setting parameters for quality control," *South African Journal of Botany*, vol. 82, no. 0, pp. 46–52, 2012.

[158] S. Shaik, N. Singh, and A. Nicholas, "HPLC and GC analyses of in vitro-grown leaves of the cancer bush *Lessertia* (*Sutherlandia*) *frutescens* L. reveal higher yields of bioactive compounds," *Plant Cell, Tissue and Organ Culture*, vol. 105, no. 3, pp. 431–438, 2011.

[159] M. Minocha, N. K. Mandava, D. Kwatra et al., "Effect of short term and chronic administration of *Sutherlandia frutescens* on pharmacokinetics of nevirapine in rats," *International Journal of Pharmaceutics*, vol. 413, no. 1-2, pp. 44–50, 2011.

[160] S. E. Shackleton, C. M. Shackleton, T. Cunningham, C. Lombard, C. A. Sullivan, and T. R. Netshiluvhi, "Knowledge on *Sclerocarya birrea* subsp. *caffra* with emphasis on its importance as a non-timber forest product in South and southern Africa: a summary. Part 1: Taxonomy, ecology and role in rural livelihoods," *Southern African Forestry Journal*, no. 194, pp. 27–41, 2002.

[161] A. P. Li, "A comprehensive approach for drug safety assessment," *Chemico-Biological Interactions*, vol. 150, no. 1, pp. 27–33, 2004.

[162] A. Fugh-Berman, "Herb-drug interactions," *The Lancet*, vol. 355, no. 9198, pp. 134–138, 2000.

[163] E. Mills, B. C. Foster, R. Van Heeswijk et al., "Impact of African herbal medicines on antiretroviral metabolism," *AIDS*, vol. 19, no. 1, pp. 95–97, 2005.

[164] N. R. Crouch, E. Douwes, M. M. Wolfson, G. F. Smith, and T. J. Edwards, "South Africa's bioprospecting, access and benefit-sharing legislation: current realities, future complications, and a proposed alternative," *South African Journal of Science*, vol. 104, no. 9-10, pp. 355–366, 2008.

Hindawi Publishing Corporation
Journal of Chemistry
Volume 2013, Article ID 352042, 7 pages
http://dx.doi.org/10.1155/2013/352042

Research Article

Analysis of A-Type and B-Type Highly Polymeric Proanthocyanidins and Their Biological Activities as Nutraceuticals

Kazushige Yokota,[1] Hideto Kimura,[2] Satoshi Ogawa,[2] and Takashi Akihiro[3]

[1] *Department of Life Science and Biotechnology, Faculty of Life and Environmental Science, Shimane University, 1060 Nishikawatsu-cho, Matsue, Shimane 690-8504, Japan*
[2] *The Department of Research and Development, Kotobuki Seika Co., Ltd., 2028 Hatagasaki, Yonago, Tottori 683-0845, Japan*
[3] *Department of Biological Science, Faculty of Life and Environmental Science, Shimane University, 1060 Nishikawatsu-cho, Matsue, Shimane 690-8504, Japan*

Correspondence should be addressed to Kazushige Yokota; yokotaka@life.shimane-u.ac.jp

Received 22 October 2012; Accepted 23 November 2012

Academic Editor: A. Hamid A. Hadi

Proanthocyanidins have a series of heteroflavan-3-ols, (+)-catechin/(−)-epicatechin units, which are linked through a single B-type linkage and a doubly linked A-type linkage. Recently, we have performed the structural characterization of seed shells of the Japanese horse chestnut and fruits of blueberry and cranberry. The molecular sizes of them were higher in the order of blueberry > cranberry > seed shells of the Japanese horse chestnut between the respective fractions. For the analysis of terminal and extension units in those proanthocyanidins, the isolated fractions were subjected to the thiolytic cleavage of the B-type linkages using 1-dodecanethiol, and the resulting degradation products were identified by ultraperformance liquid chromatography coupled with electrospray-ionization mass spectrometry. These analyses provided fast and good resolution of the degradation products and revealed higher proportions of A-type linkages compared with B-type linkages in both isolated fractions in the order of the seed shells > cranberry > blueberry. Moreover, the isolated fractions with higher molecular sizes and those more abundant in the proportions of A-type linkages were found to be more effective in the inhibition of pancreatic lipase activity. The results suggest that A-type highly polymeric proanthocyanidins are promising for the attenuation of lipid digestion as dietary supplements.

1. Introduction

Proanthocyanidins are well known to be a subclass of polyphenolic compounds that can be detectable in a wide variety of food sources, such as fruits, nuts, beans, apples, and red wine [1]. These compounds are regarded as healthy food supplements due to their activities serving as potent antioxidants to suppress the generation of reactive oxygen species under the oxidative stress in the body [2]. As well, proanthocyanidins have been shown to have other biological activities including antimicrobial, antiallergy, anticancer, and antiobesity actions. To understand the mechanism of specific effects of those compounds on the biological effects, the structural elucidation of polyphenolic polymers is a prerequisite.

Proanthocyanidins comprise a series of heteropolyflavan-3-ols, (+)-catechin/(−)-epicathechin units, which are linked through a single B-type linkage of C4 → C6 or C4 → C8, and a doubly linked A-type linkage including a C4 → C8 bond and an additional ether bond between O7 → C2 (Figure 1). In general, proanthocyanidins are classified as B-type proanthocyanidins mainly consisting of B-type linkages or A-type proanthocyanidins having higher proportion of A-type linkages in addition to the presence of B-type bonds [1, 3, 4]. Recently, we found that seed shells of the Japanese horse chestnut contained the higher levels of A-type highly polymeric proanthocyanidins [2]. Furthermore, in vitro and in vivo studies have revealed that these highly polymeric proanthocyanidins have more healthy effects to

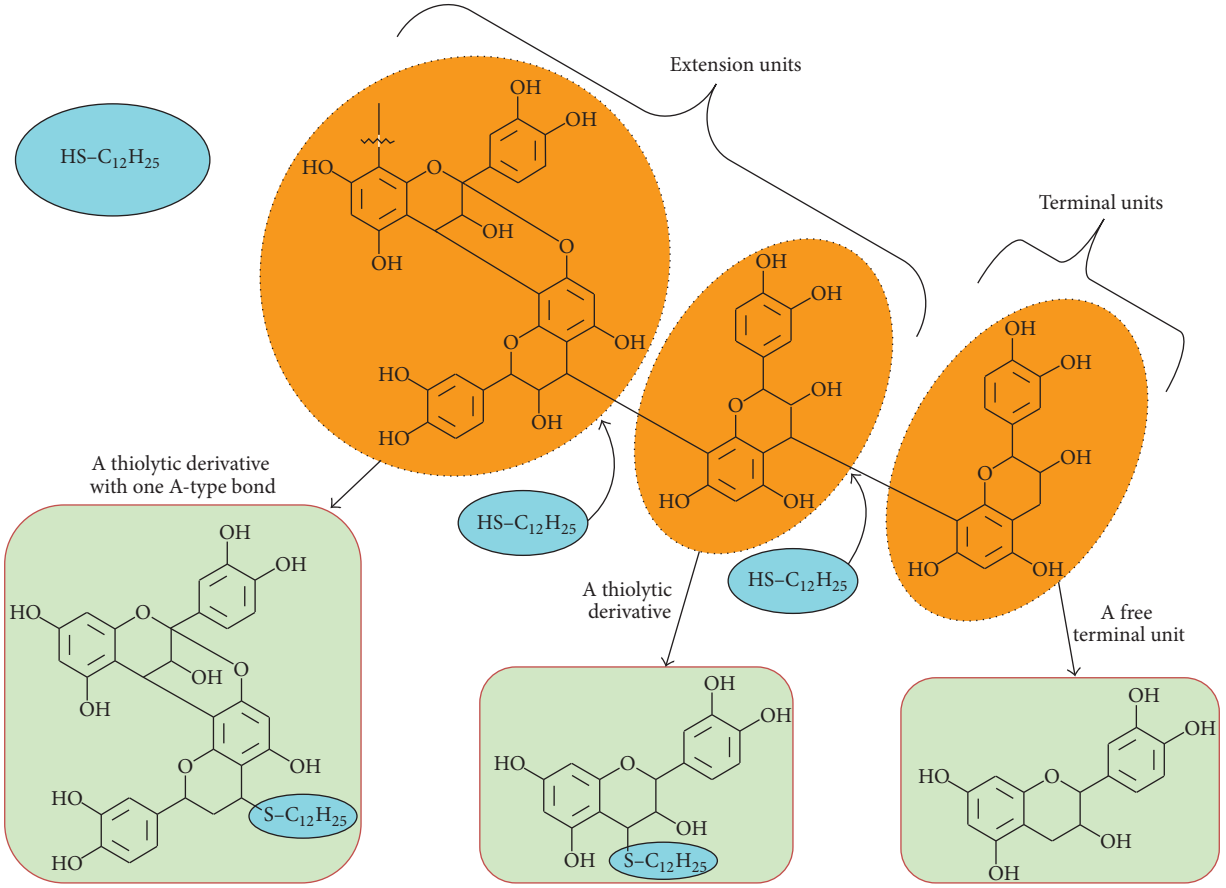


Figure 1: Thiolytic cleavage of highly polymeric proanthocyanidins having a series of heteropolyflavan-3-ols, (+)-catechin/(−)-epicatechins, with doubly linked A-type interflavan linkages and single B-type bonds.

exhibit antidiabetic [5] and antiobesity actions [6, 7] as well as their antioxidant activities [2]. However, the structural characterization of the above highly polymeric proanthocyanidins is limited because of difficulties associated with their highly polymerized forms. Hence, no detailed studies have been described to determine the proportions of terminal and extension units of A-type and B-type highly polymeric proanthocyanidins as well as their polymer sizes.

Recently, we attempted to perform structural characterization of highly polymeric proanthocyanidins from seed shells of the Japanese horse chestnut (*Aesculus turbinata* BLUME) and cranberry (*Vaccinium macrocarpon* Aiton) fruit as rich sources of A-type highly polymeric proanthocyanidins by thiolytic degradation with 1-dodecanethiol. These results were compared with those of the corresponding polymers from blueberry (*Vaccinium angustifolium* Aiton) fruit as a typical source of B-type proanthocyanidins. The thiolytic treatment is expected to cleave only single B-type bond of those proanthocyanidins without affecting the doubly linked A-type linkages, resulting in the formation of thioether derivatives of 1-dodecanethiol from extension units and free terminal units (Figure 1) [8–10]. Using chromatographic and instrumental analyses we have obtained structural information on the proportion of A-type linkages relative to B-type linkages and the molecular sizes of highly polymeric proanthocyanidins isolated differently from three types of food sources. Furthermore, we discuss the implication of different preparations of them as nutraceuticals on the basis of our recent data regarding the inhibitory effects on the digestion of carbohydrates and lipids [6, 7, 11].

2. Preparation and Isolation of Highly Polymeric Proanthocyanidins

Seeds of the Japanese horse chestnut were collected from the forest of northern Hyogo Prefecture in Japan, and frozen fruits of blueberry and cranberry were purchased from the commercial sources [2]. These food sources were used for crude extraction of total polyphenolic compounds with 70% acetone as described in our reports [2, 11]. These crude extracts from each food source were fractionated sequentially by column chromatography on Diaion HP-20 and Chromatorex ODS1024T to obtain total fraction of polyphenolic compounds. The resulting fractions were furthermore separated by column chromatography with Sephadex LH-20 into three fractions corresponding to F1, F2, and F3 by eluting with ethanol, methanol, and 70% acetone, respectively. These fractions can be separated on the basis of varied sizes of proanthocyanidins. After further analyses, we found

Table 1: Molecular sizes of two different fractions of highly polymeric proanthocyanidins isolated individually from food sources of seed shells of Japanese horse chestnut, blueberry, and cranberry as determined by gel permeation HPLC.

	Fraction	Retention time (min)	Mp
Seed shells of Japanese horse chestnut	AF2	3.14	14800
	AF3	2.90	30900
Blueberry fruit	BF2	2.96	25500
	BF3	2.81	40800
Cranberry fruit	CF2	3.07	20300
	CF3	2.87	33900

The fractions of F2 and F3 were individually obtained by column chromatography on Sephadex LH-20 from seed shells of Japanese horse chestnut (AF2 and AF3), and fruits of blueberry (BF2 and BF3) and cranberry (CF2 and CF3).

that the fraction of F2 and F3 contained highly polymeric proanthocyanidins from individual food sources while low-molecular-weight phenolic substances are only included in the F1 fraction [2]. The fractions of F2 and F3 from seed shells of the Japanese horse chestnut, blueberry, and cranberry are now referred to as AF2 and AF3, BF2 and BF3, and CF2 and CF3, respectively.

To assess the molecular sizes of highly polymeric proanthocyanidins, the fractions of F2 and F3 from individual food sources were applied to the gel permeation high-performance liquid chromatography (HPLC) on TSK-gel super AW3000 that had been calibrated with standard polystyrenes as described earlier by Bae et al. [12]. The molecular sizes of the samples were estimated by determining individual peak-top molecular weight (Mp) of standard polystyrenes (Table 1). As to the same food source, the molecular sizes of the F3 fractions were found to be always higher than those of the F2 fractions. Moreover, This analysis revealed that the molecular sizes of them were higher in the order of blueberry > cranberry > seed shells of the Japanese horse chestnut by the comparison of them between same fractions from different food sources. We utilized standard polystyrenes with different sizes for the calibration in place of standard polymeric proanthocyanidins that are not available at present. Therefore, the deviations from the estimated molecular sizes of samples could not be excluded. However, the estimation by the gel permeation chromatography is useful enough to compare the relative molecular sizes of highly polymeric proanthocyanidins from different food sources.

3. Thiolytic Cleavage of Highly Polymeric Proanthocyanidins and Their Chromatographic Analysis

Thiolytic cleavage of proanthocyanidins and the related compounds is considered to be a useful method for the analysis of components in the extension and termination units of a series of heteropolyflavan-3-ols and the estimation of the polymerization degree of them. The extension units through the single B-linkages can be cleaved by the nucleophilic attack of benzyl mercaptan [9] or 1-dodecanethiol [10] to produce the respective derivatives, whereas those with the doubly linked A-type bonds are resistant to the thiolysis. The terminal units are released as underivatized (+)-catechin or (−)-epicatechin, or oligomers having other extension units through the A-type bonds. Initially, we compared the thiolytic reactions of highly polymeric proanthocyanidins from seed shells of the Japanese horse chestnut using both benzyl mercaptan and 1-dodecanethiol. The total recovery of the initial compounds was found to be higher with 1-dodecanethiol than with benzyl mercaptan. In addition, we recognized poor resolution of the benzylthioether derivatives with the A-type linkages as the reaction products by reverse-phase HPLC. Considering these issues, we decided to employ 1-dodecanethiol for the thiolytic cleavage of highly polymeric proanthocyanidins. The resulting dodecylsulfide derivatives were separated more efficiently by our conditions of HPLC and ultra-performance liquid chromatography (UPLC).

Until now, the thiolytic products of proanthocyanidins have been analyzed by the injection to the HPLC column. Although this method can simultaneously detect both extension units as the dodecylsulfide derivatives and released terminal units, the accurate determination of the amount of the terminal units became difficult due to much higher levels of the extension units compared with the terminal units especially in highly polymerized forms. To overcome this issue, we introduced a revised method to determine the amounts of both units by chromatographic analysis after the separation of the derivatives of extension units from terminal units. For this, the phase separation with water and chloroform was effective because the dodecylsulfide derivatives of the extension units are soluble in organic phase while the terminal units are only soluble in aqueous phase [11].

4. Identification of Terminal and Extension Units in A-Type and B-Type Highly Polymeric Proanthocyanidins by Thiolytic Analysis

To obtain the structural information on the terminal and extension units in highly polymeric proanthocyanidins prepared from three types of food sources, the isolated individual fractions of F2 and F3 from different foods were used for the thiolytic cleavage. The resulting thiolytic products were subjected to the analysis by ultra-performance liquid chromatography coupled with electrospray-ionization mass spectrometry (UPLC-ESI/MS) using a Waters Synapt G2 High Definition Mass Spectrometry System (Nihon Waters,

Table 2: Proportions of terminal units in highly polymeric proanthocyanidins from seed shells of Japanese horse chestnut, blueberry, and cranberry as determined by UPLC-ESI/MS of the thiolytic products.

	Cleaved terminal unit	Proportion (mol%)					
		AF2	AF3	BF2	BF3	CF2	CF3
1	(+)-Catechin	10.2	8.9	51.8	35.8	7.8	7.8
2	(−)-Epicatechin	70.7	74.6	48.2	64.2	56.7	52.2
3	Procyanidin A2	19.1	16.5	N.D.	N.D.	35.5	40.0

The terminal units were quantified by monitoring the absorbance at 280 nm. Data represent the mean of three independent experiments. N.D.: not detectable.

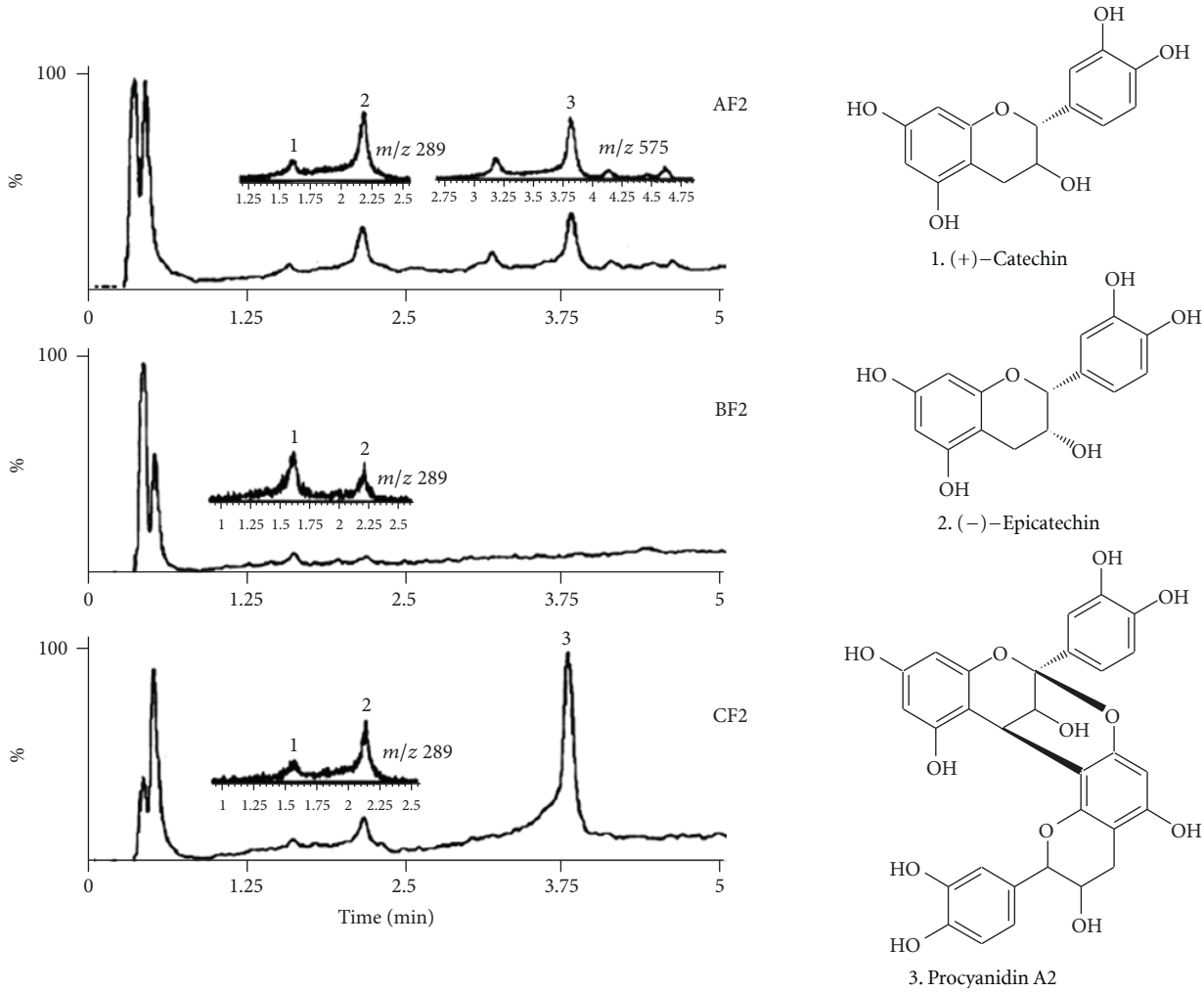


Figure 2: Analysis of terminal units of highly polymeric proanthocyanidins from three food sources using the individual fractions of F2 after thiolytic cleavage by UPLC-ESI/MS.

Tokyo, Japan) connected with an analytical UPLC column of Cosmosil 2.5C18-MS-II (75 mm × 2 mm i.d., 2.5 μm particle, Nacalai, Japan) as described [11]. Based on the mass spectral data of negative ions $[M–H]^-$ reflecting molecular ions and the product ions characteristic of individual compounds, we identified the thiolytic products of the terminal and extension units and quantified the proportion of the thiolytic products. The analysis of the terminal units revealed that all of the food sources contained the monomeric (+)-catechin (**1**) and (−)-epicatechin (**2**) (Figure 2, Table 2). In addition, procyanidin A2 (**3**) with one A-type bond was recognized in the fractions F2 and F3 from seed shells of the Japanese horse chestnut (AF2 and AF3) and cranberry fruit (CF2 and CF3), reflecting characteristic A-type proanthocyanidins. In sharp contrast, the peak of **3** was not detectable in the terminal units of both BF2 and BF3 fractions from blueberry fruit, demonstrating the typical source of B-type proanthocyanidins.

The extension units of highly polymeric proanthocyanidins can be analyzed as the dodecylsulfide derivatives after their thiolytic degradation with 1-dodecanethiol and the

Table 3: Proportions of extension units in highly polymeric proanthocyanidins from seed shells of Japanese horse chestnut, blueberry, and cranberry as determined by UPLC-ESI/MS of the dodecylsulfide derivatives after thiolytic cleavage.

	Cleaved extension unit	Proportion (mol%)					
		AF2	AF3	BF2	BF3	CF2	CF3
4	(Epi)gallocatechin dodecylsulfide	1.9	1.7	1.5	3.1	7.6	6.3
5	(Epi)gallocatechin-(epi)catechin A-type dimer dodecylsulfide	3.6	2.8	N.D.	N.D.	N.D.	N.D.
6	(Epi)catechin A-type trimer dodecylsulfide	0.9	0.8	N.D.	N.D.	N.D.	N.D.
7	(Epi)catechin A-type dimer dodecylsulfide	32.2	25.3	6.8	6.4	25.4	20.3
8	(Epi)catechin dodecylsulfide	61.3	69.4	91.6	90.6	67.0	73.3

The dodecylsulfide derivatives of extension units were quantified by monitoring the absorbance at 280 nm. Data represent the mean of three independent experiments. N.D.: not detectable.

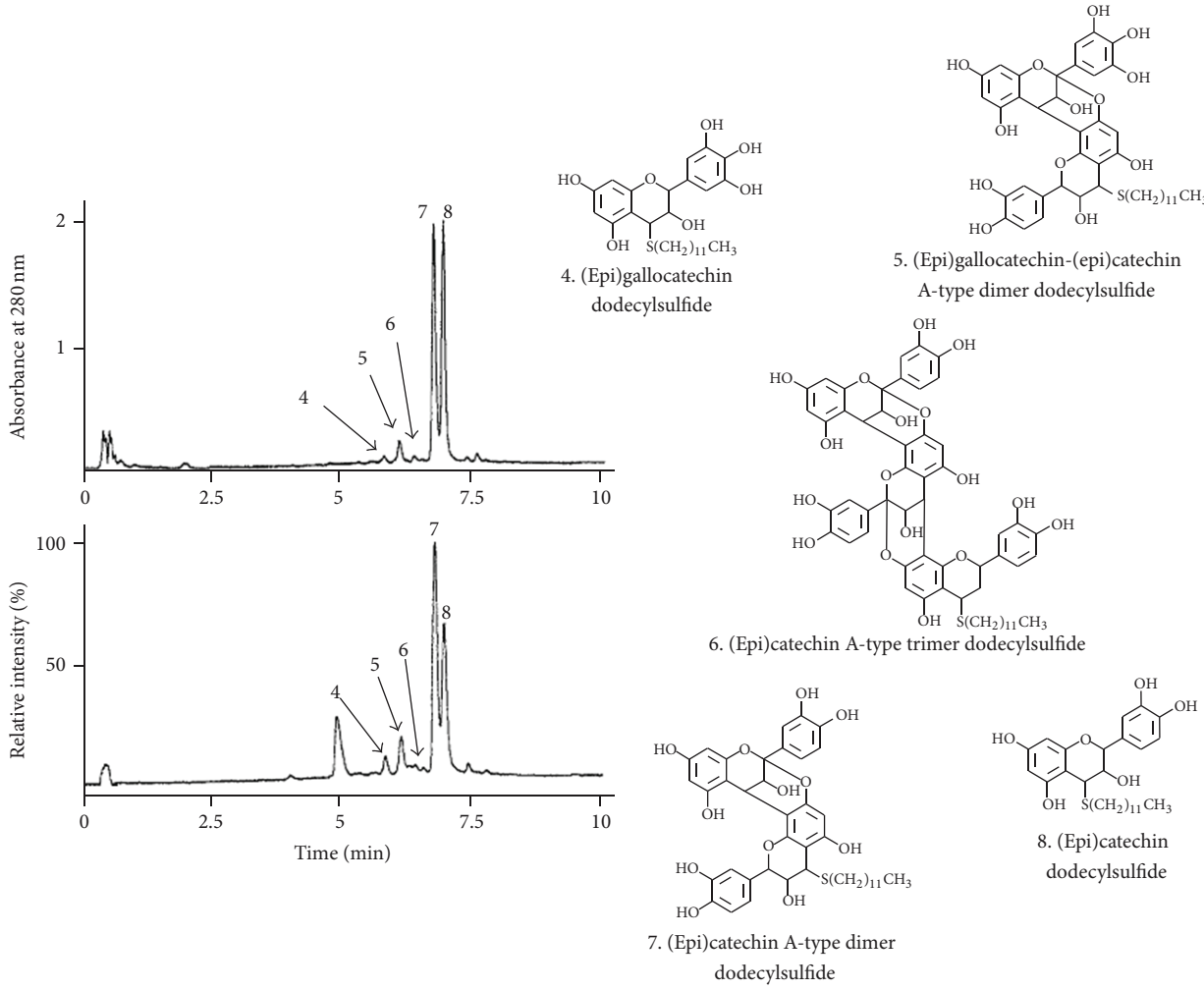


Figure 3: Analysis of extension units of the AF2 fraction of highly polymeric proanthocyanidins from seed shells of Japanese horse chestnut as the dodecylsulfide derivatives after thiolytic cleavage by UPLC-ESI/MS.

identification by UPLC-ESI/MS (Figure 3, Table 3). The analysis of the fractions of F2 and F3 from all the food sources revealed that (epi)catechin dodecylsulfide (**8**) are present as the most abundant thiolytic product. Higher proportions of it were found in the fractions of blueberry fruit, reflecting typical B-type proanthocyanidins. In addition, (epi)catechin A-type dimer dodecylsulfide (**7**) were commonly detected as the secondly predominant cleaved products from all the fractions. The higher proportions of it was detectable in the order of the seed shells of the Japanese horse chestnut > cranberry fruit > blueberry fruit. These findings indicate much more abundant proportions of A-type linkages in the fractions from the seed shells of the Japanese horse chestnut (AF2 and AF3) and cranberry frit (CF2 and CF3)

than those from blueberry fruit (BF2 and BF3). As well, the fractions of AF2 and AF3 contained other thiolytic products of (epi)gallocatechin-(epi)catechin A-type dimer dodecylsulfide (**5**) and (epi)catechin A-type trimer dodecylsulfide (**6**) as typical A-type proanthocyanidins. Interestingly, (epi)gallocatechin dodecylsulfide (**4**) was detectable in the fractions of F2 and F3 from all the food sources and more abundant in the fractions from cranberry fruit (CF2 and CF3). With respect to the comparisons between the F2 and F3 fractions from individual food sources, we found no marked differences between them. Taken together, our analysis provided the evidence that seed shells of the Japanese horse chestnut contained apparently higher proportions of doubly liked A-type linkages with variations in the binding mode than fruits of blueberry and cranberry.

Earlier, the thiolytic cleavage of oligomeric procyanidins with only B-type linkages have been accomplished using benzylmercaptan to calculate the mean degree of polymerization after the quantification of those derivatives and monomeric flavan-3-ols [9]. On the other hand, highly polymeric proanthocyanidins with both A-type and B-type linkages are more complicated to analyze it due to the lack of the authentic compounds corresponding to thiolytic products and much higher proportion of the extension units compared with the terminal units. We tried to estimate the average degree of polymerization by the determination of the thiolysis of our highly polymeric proanthocyanidins. However, the calculated values did not match the molecular sizes estimated by the gel permeation chromatography. These erroneous results could be partly explained by the more preferential degradation of terminal units during the extended reaction of thiolytic cleavage. Hence, more extensive efforts to overcome these issues associated with the reaction conditions should be done for the accurate estimation of the polymerization degree of highly polymeric proanthocyanidins by thiolytic cleavages.

5. Biological Activities of A-Type and B-Type Highly Polymeric Proanthocyanidins as Neutraceuticals

Excess consumption of high-fat diets is well known to lead to the development of obesity, which is a major risk factor for the onset of insulin resistance and the increased morbidity of diabetes mellitus and cardiovascular disease. Therefore, much attention has been paid to the neutraceuticals that have antiobesity effects. Recently, we have described that highly polymeric pronanthocyanidins from seed shells of the Japanese horse chestnut can inhibit pancreatic lipase in a dose-dependent manner in vitro assay system [6]. The effectiveness of these isolated fractions in suppressing the digestion and absorption of lipids was furthermore confirmed by in vivo oral fat tolerance test in mice [6] and long-term antiobesity effects in obese mice fed a high-fat diet supplemented with the total fraction as a drink [7].

More recently, we have isolated the fractions of F2 and F3 from highly polymeric proanthocyanidins of three types of food sources and tested the individual fractions for their inhibitory effects on the enzymatic activity of pancreatic lipase [11]. By analyzing the dose-response curves of the inhibitory actions, w noticed that the F3 fractions with higher molecular sizes from all the sources had totally more potent effects to inhibit pancreatic lipase than the F2 fractions with smaller molecular ones from the same sources. This observation indicates that higher degree of polymerization is one of the important factors to show the inhibitory effect. Moreover, these results from different food sources were compared between the same fractions of F2 or F3 to obtain the structural information relevant to the difference in the biological activities of those fractions. The comparison between the F2 fractions from different sources revealed that the AF2 fraction with the highest proportions of doubly linked A-type linkages exhibited the most potent effects to inhibit pancreatic lipase although the molecular sizes are the smallest among the fractions of F2 from three sources [11]. However, no significant difference was observed between the F3 fractions from different food sources, presumably due to much higher molecular sizes and more inhibitory effects of the F3 fractions than those of the F2 fractions. Taking these findings into consideration, both the higher molecular sizes and more abundance of A-type linkages of polymeric proanthocyanidins should be crucial determinants that contribute to more effective inhibition of pancreatic lipase. Consistent with this indication, we demonstrated that procyanidin A2 with one A-type linkage had much more potent activity to suppress pancreatic lipase than procyanidin B2 with one B-type bond.

Highly polymeric proanthocyanidins are generally considered to bind to a number of proteins nonspecifically. However, their effects to inhibit pancreatic lipase are not explained simply by the absorption to the pancreatic lipase protein because the enzyme was more potently inhibited by AF2 with higher levels of doubly linked A-type linkages than other related BF2 and CF2 in spite of the lowest size of AF2 among the F2 fractions [11]. Moreover, we have recently examined the effects of highly polymeric proanthocyanidins on the digestive enzymes of carbohydrates, including α-amylase, maltase, and sucrase [5]. According to the results regarding their dose-dependence, the fractions of AF2 and AF3 from seed shells of the Japanese horse chestnut were found to be much more effective to suppress the enzyme activity of α-amylase compared with other α-glycosidases. Considering these findings, these highly polymeric polymers should have the specificity to inhibit digestive enzymes, depending on the proportions of A-type and B-type linkages as well as the degree of the polymerization.

Earlier, Nakai et al. has reported the importance of galloyl moieties in polymerized polylphenolic compounds from oolog tea in the inhibition of pancreatic lipase [13]. We also noticed that (−)-epigallocatechin 3-O-gallate had much potent effect to inhibit pancreatic lipase in our assay conditions [6]. Since galloyl moieties were detected by the thiolytic cleavage of our fractions from highly polymeric proanthocyanidins, this galloyl group serves as an inhibitor of pancreatic lipase. As separate biological activities, A-type proanthocyanidins have been described to exhibit bacterial antiadhesion activity [3] and anticancer effects [14]. We expect further studies to find the unique and promising

biological activity of doubly linked A-type bonds and other moieties in proanthocyanidins and the related natural products.

6. Conclusions

Recently, we have performed the structural characterization of highly polymeric proanthocyanidins from three food sources including seed shells of the Japanese horse chestnut and fruits of blueberry and cranberry. Thiolytic cleavage of the single B-type linkages with 1-dodecanethiol resulted in the better resolution of the cleaved products with the doubly linked A-type bonds or the dodecylsulfide derivatives by HPLC and UPLC-ESI/MS. Thus, we were able to determine the proportions of the terminal and extension units of the isolated fractions. The fractions with higher molecular sizes and higher proportions of A-type linkages over B-type bonds exerted more potent effects to inhibit pancreatic lipase.

References

[1] R. L. Prior and L. Gu, "Occurrence and biological significance of proanthocyanidins in the American diet," *Phytochemistry*, vol. 66, no. 18, pp. 2264–2280, 2005.

[2] S. Ogawa, H. Kimura, A. Niimi, T. Katsube, M. Jisaka, and K. Yokota, "Fractionation and structural characterization of polyphenolic antioxidants from seed shells of Japanese horse chestnut (*Aesculus turbinata* BLUME)," *Journal of Agricultural and Food Chemistry*, vol. 56, no. 24, pp. 12046–12051, 2008.

[3] A. B. Howell, J. D. Reed, C. G. Krueger, R. Winterbottom, D. G. Cunningham, and M. Leahy, "A-type cranberry proanthocyanidins and uropathogenic bacterial anti-adhesion activity," *Phytochemistry*, vol. 66, no. 18, pp. 2281–2291, 2005.

[4] M. A. Kelm, J. F. Hammerstone, and H. H. Schmitz, "Identification and quantitation of flavanols and proanthocyanidins in foods: how good are the datas?" *Clinical and Developmental Immunology*, vol. 12, no. 1, pp. 35–41, 2005.

[5] S. Ogawa, H. Kimura, A. Niimi, M. Jisaka, T. Katsube, and K. Yokota, "Inhibitory effects of polyphenolic compounds from seed shells of Japanese horse chestnut (*Aesculus turbinata* Blume) on carbohydrate-digesting enzymes," *Nippon Shokuhin Kagaku Kogaku Kaishi*, vol. 56, no. 2, pp. 95–102, 2009.

[6] H. Kimura, S. Ogawa, A. Niimi, M. Jisaka, T. Katsube, and K. Yokota, "Inhibition of fat digestion by highly polymeric proanthocyanidins from seed shells of Japanese horse chestnut (*Aesculus turbinata* Blume)," *Nippon Shokuhin Kagaku Kogaku Kaishi*, vol. 56, no. 9, pp. 483–489, 2009.

[7] H. Kimura, S. Ogawa, A. Sugiyama, M. Jisaka, T. Takeuchi, and K. Yokota, "Anti-obesity effects of highly polymeric proanthocyanidins from seed shells of Japanese horse chestnut (*Aesculus turbinata* Blume)," *Food Research International*, vol. 44, no. 1, pp. 121–126, 2011.

[8] S. Guyot, N. Marnet, and J.-F. Drilleau, "Thiolysis-HPLC characterization of apple procyanidins covering a large range of polymerization states," *Journal of Agricultural and Food Chemistry*, vol. 49, no. 1, pp. 14–20, 2001.

[9] L. Gu, M. Kelm, J. F. Hammerstone et al., "Fractionation of polymeric procyanidins from lowbush blueberry and quantification of procyanidins in selected foods with an optimized normal-phase HPLC-MS fluorescent detection method," *Journal of Agricultural and Food Chemistry*, vol. 50, no. 17, pp. 4852–4860, 2002.

[10] M. Kusuda, K. Inada, T. O. Ogawa et al., "Polyphenolic constituent structures of *Zanthoxylum piperitum* fruit and the antibacterial effects of its polymeric procyanidin on methicillin-resistant *Staphylococcus aureus*," *Bioscience, Biotechnology and Biochemistry*, vol. 70, no. 6, pp. 1423–1431, 2006.

[11] H. Kimura, S. Ogawa, T. Akihiro, and K. Yokota, "Structural analysis of A-type or B-type highly polymeric proanthocyanidins by thiolytic degradation and the implication in their inhibitory effects on pancreatic lipase," *Journal of Chromatography A*, vol. 1218, no. 42, pp. 7704–7712, 2011.

[12] Y. S. Bae, L. Y. Foo, and J. J. Karchesty, "GPC of natural procyanidin oligomers and polymers," *Holzforschung*, vol. 48, no. 1, pp. 4–6, 1994.

[13] M. Nakai, Y. Fukui, S. Asami et al., "Inhibitory effects of oolong tea polyphenols on pancreatic lipase in vitro," *Journal of Agricultural and Food Chemistry*, vol. 53, no. 11, pp. 4593–4598, 2005.

[14] C. C. Neto, "Cranberry and its phytochemicals: a review of in vitro anticancer studies," *Journal of Nutrition*, vol. 137, no. 1, pp. 186s–193s, 2007.

Hindawi Publishing Corporation
Journal of Chemistry
Volume 2013, Article ID 561905, 7 pages
http://dx.doi.org/10.1155/2013/561905

Research Article

The Inhibitory Effects of Aqueous Extract from Guava Twigs, *Psidium guajava* L., on Mutation and Oxidative Damage

Zhi-Chyang Kang,[1] Ming-Tsung Yen,[2] Chih-Kwang Chiu,[3] Horng-Cherng Wu,[3] Shu-Ling Huang,[4] Shan-Pao Tai,[2] and Bor-Sen Wang[2,3]

[1] *Department of Health and Nutrition, Chia-Nan University of Pharmacy and Science, No. 60 Erh-Jen Rd., Sec.1, Tainan 717, Taiwan*
[2] *Department of Applied Life Science and Health, Chia-Nan University of Pharmacy and Science, 60 Erh-Jen Road, Section 1, Tainan 717, Taiwan*
[3] *Department of Food Science and Technology, Chia-Nan University of Pharmacy and Science, 60 Erh-Jen Road, Section 1, Tainan 717, Taiwan*
[4] *Department of Food Nutrition, Chung Hwa University of Medical Technology, 89 Wenhwa 1st Street, Tainan 717, Taiwan*

Correspondence should be addressed to Bor-Sen Wang; wangbs@mail.chna.edu.tw

Received 29 June 2012; Accepted 5 November 2012

Academic Editor: Mehmet Emin Duru

This study examines the inhibitory effects of the aqueous extract from guava twigs (GTE), *Psidium guajava* L., on mutation and oxidative damage. The results show that GTE inhibits the mutagenicity of 4-nitroquinoline-N-oxide (4-NQO), a direct mutagen, and 2-aminoanthracene (2-AA), an indirect mutagen, toward *Salmonella typhimurium* TA 98 and TA 100. In addition, GTE shows radical scavenging, reducing activities, tyrosinase inhibition, and liposome protection effects. Meanwhile, GTE in the range of 0.1–0.4 mg/mL protects liver cells from *tert*-butyl-hydroperoxide-(*t*-BHP-) induced cytotoxicity. Furthermore, the cytotoxicity inhibition of GTE in the *t*-BHP-treated cells was demonstrated in a dose-dependent manner. High-performance liquid chromatography analysis suggests that the major phenolic constituents in GTE are gallic acid, ferulic acid, and myricetin. These active phenolic components may contribute to the biological protective effects of GTE in different models. The data suggest that GTE exhibiting biological activities can be applied to antimutation, antityrosinase, and antioxidative damage.

1. Introduction

The production of DNA damage produces a domino effect on mutation and aging diseases. Various mutagens present on food increase oxidative stress and cancer risk through different mechanisms in cells. For example, 4-nitroquinoline-N-oxide (4-NQO), a direct and strong mutagen, is a quinoline derivative carcinogen and can also induce potent intracellular production of oxidative stress [1]. In addition, 2-aminoanthracene (2-AA), a known carcinogenic polycyclic aromatic amine, can induce tumors primarily in the liver [2]. In Ames test, 2-AA requires metabolic activation by the S9 liver preparation. The metabolic products of these mutagens chiefly bind to DNA at guanine residues. The consequence is that whenever these DNA adducts are formed, DNA mutation may increase, eventually increasing the risk of tumor progression.

Other than DNA damage, these mutagens may be metabolized and induce harmful oxidative stress in cells, which also destroys the biological molecules (e.g., lipids) and causes mutations [3]. However, many reports suggest that intracellular oxidative stress derived from reactive nitrogen species (RNS) and reactive oxygen species (ROS) arises during physiological metabolism and after exposure to various chemical encouragements. Oxidative stress can be observed in different pathological states, such as atherosclerosis and cancer. For example, tyrosinase (EC 1.14.18.1) plays an important role in the browning reaction. This enzymatic browning may result in discolouration and reduce the nutritional value of food. In order to prevent the browning, use of food additives including

reducing agent and enzyme inhibitor (such as sulphite) has been recognized [4]. During this complex process, the generation of *o*-quinone will further diminish glutathione and decrease the antioxidant capacity in cells. In the recent approach, the tyrosinase inhibitor from natural sources has become popular not only as a food additive to prevent the browning but also in applied products to decrease oxidative stress.

Guava (*Psidium guajava* L.), a genus of the Myrtaceae family, is a commercially available food crop that is distributed widely in tropical and subtropical regions around the world, such as Taiwan, Vietnam, Thailand, and China. It is a popular fruit with consumers all over the world. It has been used for treating a number of diseases such as inflammation [5], hepatotoxicity [6], and hypertension [7]. Many studies suggested that the fruits and leaves of guava contain bioactive compounds, such as phenolic acids and flavonoids [8]. This is the first study to determine the biological effects of guava twigs, an agricultural waste material, on mutation and tyrosinase activity. The objective of this work is to determine the antimutation and antioxidative damage activity of aqueous extract from guava twigs.

2. Materials and Methods

2.1. Materials. 4-Nitroquinoline-N-oxide (4-NQO), 2-aminoanthracene (2-AA), thiobarbituric acid (TBA), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), *tert*-butylhydroperoxide (*t*-BHP), and mushroom tyrosinase were purchased from Sigma-Aldrich (St. Louis, MO, USA). L-3,4-Dihydroxyphenylalanin (L-DOPA) was obtained from Acros Organic (Geel, Belgium). Culture medium and top agar were prepared as previously described [9]. The twigs of guava were harvested from commercial plantations in Tainan, Taiwan during July 2011.

2.2. Sample Preparation. The guava twigs were grounded after dried in oven at 50°C for 24 h. The powder (100 g) was extracted with water (1000 mL) at 100°C for 30 min and then centrifuged at 10,000 g for 20 min. The extract was filtered and the residue was reextracted under the same conditions. The combined filtrate was then freeze-dried. The yield obtained was 8.1% (w/w). The final sample was named as GTE (the aqueous extract of guava twigs).

2.3. Total Polyphenolics Assay. Total polyphenolics were determined as gallic acid equivalents [10]. The different concentrations of GTE were added to a 10 mL volumetric flask, to which 2 mL sodium carbonate (20% (w/v)) was added. After 5 min, 0.1 mL Folin-Ciocalteu reagent (50% (v/v)) was added and the volume was made up to 10 mL with H_2O. After 1 h incubation at 30°C, the absorbance was measured at 750 nm and compared to a gallic acid calibration curve.

2.4. Total Flavonoid Assay. 1 mL of GTE was incubated with 0.1 mL (2-aminoethyl) diphenyl borate (0.2% in ethanol). After 20 min of incubation, the absorbance was measured at 405 nm. The absorbance of rutin solutions was detected under the same conditions. The amount of flavonoids in GTE (in rutin equivalents) was calculated [11].

2.5. ABTS Radical Cation Inhibition Assay. This assay determined the capacity of GTE to scavenge the ABTS radical cation. The ABTS radical cation scavenging activity was measured as previously described [12]. The ABTS radical cation was generated by reacting 1 mM ABTS with 0.5 mM hydrogen peroxide and 10 units/mL horseradish peroxidase in the dark at 30°C for 2 h. After 1 mL ABTS radical cation was added to samples, the absorbance at 734 nm was recorded after 10 min.

2.6. Reducing Activity Assay. The reducing power of GTE was determined as previously described [13]. Various concentrations of GTE were added to potassium ferricyanide (2.5 mL, 10 mg/mL), and the mixture was incubated at 50°C for 20 min. TCA (2.5 mL, 100 mg/mL) was added to the mixture, which was then centrifuged at 650 g for 10 min. The supernatant (2.5 mL) was mixed with distilled water (2.5 mL) and ferric chloride (0.5 mL, 1.0 mg/mL), and then the absorbance was read at 700 nm. The reducing activity was calculated against an ascorbic acid calibration curve.

2.7. Liposome Oxidation Assay. A solution containing the lecithin (580 mg) and phosphate buffer (58 mL, 10 mM, pH 7.4) was sonicated by an ultrasonic cleaner (Branson 8210, Branson Ultrasonic Corporation, Danbury, CT, USA) in an ice-cold water bath for 2 h. The sonicated solution, $FeCl_3$, ascorbic acid, and various concentrations of GTE (0.2 mL) were mixed to produce a final mixture with a concentration of 3.12 μM $FeCl_3$ and 125 μM ascorbic acid and were incubated at 37°C for 1 h. The levels of liposome oxidation were determined as previously described [9].

2.8. Mushroom Tyrosinase Activity Assay. The mushroom tyrosinase was used for the bioassay. The tyrosinase inhibitory activity was determined with the degree of inhibition on tyrosinase-catalyzed oxidation of L-DOPA as previously described [14]. All the experiments were performed in sodium phosphate buffer (pH 6.8) at 25°C. The reaction mixture consisting of 0.1 mL of GTE, 0.1 mL of mushroom tyrosinase (1000 Unit/mL) and L-DOPA (3.8 mM) was determined the absorbance at 475 nm for 5 min. The value in the absence of samples was represented as the control. The inhibition of tyrosinase activity was calculated with the following formula:

$$\text{inhibition}\,(\%) = \left(1 - \left(\frac{\text{OD}_{475}\ \text{in sample}}{\text{OD}_{475}\ \text{in control}}\right)\right) \times 100\%. \quad (1)$$

2.9. HepG2 Cells Viability Assay. HepG2 cells were purchased from Bioresources Collection and Research Center (Shin-Chu, Taiwan) and cultured in minimum essential medium (MEM) containing 10% fetal bovine serum and maintained in humidified 5% CO_2/95% air at 37°C. After cells were

cultured with samples for 2 h, in the presence of 0.2 mM *t*-BHP or not, cell viability was determined by the MTT assay [9].

2.10. Mutagenicity Assay. The mutagenicity of GTE was tested according to the Ames test with a 20 min first incubation at 37°C [15]. The histidine-requiring strains of *Salmonella typhimurium* TA 98 and TA 100 were obtained from Taiwan Agricultural Chemicals and Toxic Substances Research Institute (Taichung, Taiwan). The external metabolic activation system, S9 mix (Molecular Toxicology, Inc., Boone, NC, USA), was prepared from Sprague-Dawley male rats treated with Aroclor 1254. Samples (0.1 mL, 20–100 mg/mL corresponding to 2–10 mg/plate) were added to the overnight cultured *S. typhimurium* TA98 or TA 100 (0.1 mL) and S9 mix (0.5 mL) or 0.1 M phosphate buffer (0.5 mL, pH 7.4) in place of the S9 mix. The entire mixture was incubated at 37°C for 20 min before molten top agar (2.0 mL) was added and then spread out in a Petri dish containing 20 mL of minimum agar. The mixture was counted after incubating at 37°C for 48 h. The toxic effects of GTE on *S. typhimurium* TA 98 and TA 100 was determined as previously described [9].

2.11. Antimutagenic Activity Assay. The antimutagenic activity of GTE was assayed according to the Ames method except for the addition of mutagen before incubation [15]. The concentrations of mutagens were tested as in a previous study [16]. The mutagens used were 4-NQO (0.5 μg/plate), a direct mutagen and 2-AA (2.5 μg/plate), which required S9 mix for metabolic activation. Mutagen (0.1 mL) was added to the mixture of a strain (TA 98 or TA 100), and samples were added with the S9 mix for 2-AA or with phosphate buffer (0.1 M, pH 7.4) for 4-NQO. The mutagenicity of each mutagen in the absence of samples is defined as 100%. The number of spontaneous revertants in the absence of mutagens and samples was used as reference. The inhibition (%) of mutagenicity of the sample was calculated as following:

$$\text{inhibition (\%)} = \left\{1 - \left[\frac{(\text{no. of his}^+ \text{ revertants with mutagen and sample} - \text{no. of spontaneous revertant})}{(\text{no. of his}^+ \text{ revertants with mutagen} - \text{no. of spontaneous revertant})}\right] \times 100\right\}. \quad (2)$$

2.12. High-Performance Liquid Chromatography (HPLC) Assay. HPLC was performed with a Hitachi Liquid Chromatograph (Hitachi Ltd., Tokyo, Japan), consisting of two model L-7100 pumps, and one model L-7455 photodiode array detector. Sample (10 mg/mL) was filtered through a 0.45 μm filter and injected into the HPLC column. The injection volume was 20 μL and the flow rate was 0.8 mL/min. The separation temperature was 25°C. The column was a Mightysil RP-18 GP (5 μm, 250 × 4.6 mm I.D.; Kanto Corporation, Portland, OR, USA). The method involved the use of a binary gradient with mobile phases was previously described [9]. The plot of the peak-area (y) versus concentration (x, μg/mL), the regression equations of the three marker compounds, and their correlation coefficients (r) were as follows: gallic acid, $y = 0.0161x + 0.0102$ ($r^2 = 0.9983$); ferulic acid, $y = 0.0264x + 0.0625$ ($r^2 = 0.9996$); myricetin, $y = 0.0512x + 0.0656$ ($r^2 = 0.9991$).

2.13. Statistical Analysis. All data were presented as means ± standard deviations (SD). Statistical analysis involved use of the Statistical Analysis System software package (SAS Institute Inc.). Analysis of variance was performed by ANOVA procedures. Significant differences between means were determined by Duncan's multiple range tests at a level of $P < 0.05$.

3. Results

The HPLC chromatographic analysis showed that bioactive phenolic components in GTE have been identified as gallic acid, ferulic acid, and myricetin by measuring their retention time and UV absorbance, in relation to standards (Figure 1). Thus, gallic acid, ferulic acid, and myricetin were selected as marker compounds for the HPLC fingerprint chromatograph of GTE.

Table 1 shows the concentrations of the three marker compounds in GTE. The relative amounts of these compounds found in GTE were in the order gallic acid (11.1 mg/g of GTE) > ferulic acid (9.4 mg/g of GTE) > myricetin (3.9 mg/g of GTE). The total levels of polyphenols and flavonoids in GTE, determined as gallic acid and rutin equivalents, were 171.2 ± 8.6 and 27.2 ± 2.1 mg/g of GTE, respectively.

The effects of GTE and the three marker components on radical scavenging and reducing activities are shown in Table 2. The reducing activity of natural products is regarded as their hydrogen donating capacity. The reducing ability of GTE and its three marker components were determined in comparison with ascorbic acid. In the range 0.01–0.04 mg/mL, GTE exhibited a reducing effect that increased, as the extract concentration increased. The reducing capacity of GTE at 0.04 mg/mL was equivalent to 15.9 μg/mL of ascorbic acid. Among the three marker components, gallic acid exhibited a better reducing activity than ferulic acid and myricetin did. The scavenging of ABTS cation radicals is a popular method for determining the antioxidant capacities of natural products. The radical scavenging activity of 0.01–0.04 mg/mL concentrations of GTE was 95.1–99.3%, indicating that GTE was a strong radical terminator. Table 2 also shows the inhibitory effects of gallic acid, ferulic acid and myricetin on radicals being 99.7%, 99.5%, and 99%, respectively. These data indicated that the three marker constituents of GTE might play a part in the antioxidant activity as well as the reducing activity of GTE.

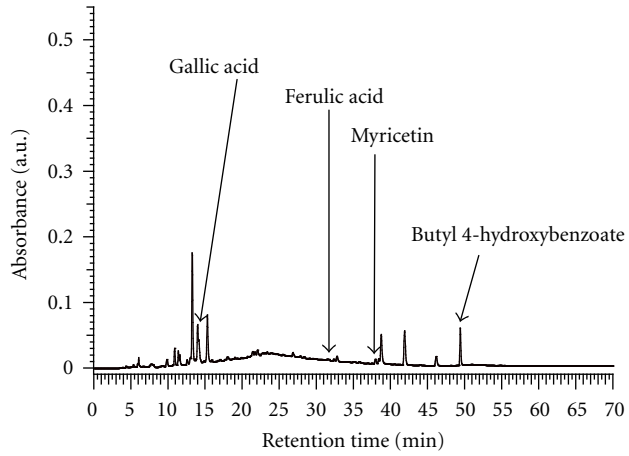


Figure 1: HPLC chromatograms of the aqueous extract of guava twigs (GTE).

Table 1: HPLC analysis of the three marker components in the aqueous extract of guava twigs (GTE).

Components	Retention time (min)	λ_{max} (nm)	Contents (mg/g GTE)
Gallic acid	13.85	218.6	11.1
Ferulic acid	31.95	233.6	9.4
Myricetin	38.06	253.1	3.9

Table 2: Effects of the aqueous extract of guava twigs (GTE) on radical scavenging and reducing activity.

Sample	(mg/mL)	Reducing activity (μg Vit C/mL)	ABTS inhibition (%)
GTE	0.01	4.4 ± 0.3	95.1 ± 0.3
	0.02	8.0 ± 0.6	98.6 ± 0.3
	0.04	15.9 ± 1.9	99.3 ± 0.3
Gallic Acid	0.01	10.8 ± 0.4	99.7 ± 1.0
Ferulic Acid	0.01	3.8 ± 0.1	99.5 ± 0.8
Myricetin	0.01	1.7 ± 0.1	99.0 ± 0.5

Data represent means ± SD for $n = 3$.

Lipid oxidation can increase cellular damage and produce toxic metabolites. In this study, liposome protection was used as a measure of decreasing lipid oxidation provided by GTE. The lower concentrations of GTE (0.01–0.04 mg/mL) did not show obvious effects in the tests of liposome protection, tyrosinase activity, and HepG2 cells viability. Therefore, the higher concentrations of GTE (0.1–0.4 mg/mL) were chosen for further tests. As shown in Table 3, GTE in concentrations of 0.1–0.4 mg/mL exhibited a 92.3–98.4% inhibitory effect on liposome oxidation induced by the Fe^{3+}/H_2O_2 system. Meanwhile, the inhibitory effects of gallic acid, ferulic acid, and myricetin on lipid oxidation were 42.5%, 32.6%, and 42.8%, respectively. These data indicated that GTE and the three marker components provided effective protection from lipid oxidation *in vitro*. Further, as shown in Table 3, GTE also had a potent inhibitory effect on mushroom

Table 3: Effects of the aqueous extract of guava twigs (GTE) on tyrosinase inhibition and liposome protection.

Sample	(mg/mL)	Tyrosinase inhibition (%)	Liposome protection (%)
GTE	0.1	17.7 ± 0.5	92.3 ± 0.9
	0.2	43.8 ± 2.9	96.5 ± 0.9
	0.4	65.6 ± 1.5	98.4 ± 3.4
Gallic acid	0.01	36.9 ± 0.6	42.5 ± 2.4
Ferulic acid	0.01	30.1 ± 2.0	32.6 ± 2.7
Myricetin	0.01	49.4 ± 2.3	42.8 ± 3.7

Data represent means ± SD for $n = 3$.

tyrosinase activity. For concentrations of 0.1–0.4 mg/mL, GTE exhibited 17.7–65.6% inhibition of tyrosinase. In this test, the inhibition of tyrosinase by GTE increased, as the sample concentration increased. Gallic acid, ferulic acid, and myricetin at 0.01 mg/mL, respectively, showed 36.9%, 30.1%, and 49.4% inhibitory effects on tyrosinase activity. Myricetin showed a greater antityrosinase effect than the other two constituents did.

Furthermore, the protective effects of GTE on *t*-BHP-induced oxidative damage in liver HepG2 cells were examined. *t*-BHP is a chemical toxin to produce peroxide intermediates in cells and promotes lipid peroxidation to result in cellular injury. Table 4 shows the protection provided by GTE from cell toxicity in *t*-BHP-induced cytotoxicity. It was found that *t*-BHP at 0.2 mM decreased cell viability down to 25.5% of control group. In the range of 0.1–0.4 mg/mL, GTE protected liver HepG2 cells against oxidative damage in a dose-dependent manner. At 0.4 mg/mL, GTE increased cell viability up to 46.1% of control group in the presence of *t*-BHP. Gallic acid, ferulic acid, and myricetin at 0.01 mg/mL, respectively, increased cell viability up to 51.9%, 58.4%, and 32.1% of control group in the presence of *t*-BHP. These data suggested that the cellular protection of GTE could attribute to its three marker components.

The Ames assay is a common method for determining the mutagenicity of natural products. In the mutation study, if a lethal toxicity occurs in a test treated sample, the results of the mutagenicity could be compromised and the numbers of revertants of TA 98 and TA 100 would be inaccurate. In this study, GTE (2–10 mg/plate) did not show any toxicity against TA 98 or TA 100 (data not shown). The mutagenicity of GTE was determined by comparing the ratio of induced revertants to spontaneous revertants, in the plates. Table 5 shows that GTE (2–10 mg/plate) did not significantly ($P > 0.05$) increase the number of colonies in *S. typhimurium* TA 98 and TA 100, with or without S9 activation.

Furthermore, the antimutagenicity of GTE on 4-NQO and 2-AA induced mutation in *S. typhimurium* TA 98 and TA 100 was examined. As shown in Table 6, GTE displayed dose-dependent protection against 4-NQO induced mutagenicity in *S. typhimurium* TA 98 and TA 100, without S9 activation. GTE at levels of 2–10 mg/plate showed 30–87% inhibition of 4-NQO induced mutagenicity in TA 98 and 31–74% inhibition in TA 100. Table 6 also shows the antimutagenicity of

Table 4: Effects of the aqueous extract of guava twigs (GTE) on *t*-butyl-hydroperoxide-(*t*-BHP-) induced HepG2 cytotoxicity.

Treatment	Sample (mg/mL)	HepG2 cell viability (% of control)
t-BHP	0	25.5 ± 8.6
	0.1	29.4 ± 5.1
t-BHP + GTE	0.2	32.6 ± 2.3
	0.4	46.1 ± 4.3
t-BHP + Gallic acid	0.01	51.9 ± 3.2
t-BHP + Ferulic acid	0.01	58.4 ± 4.2
t-BHP + Myricetin	0.01	32.1 ± 3.8

Data represent means ± SD for $n = 3$.

Table 5: The mutagenicity of the aqueous extract of guava twigs (GTE) toward *S. typhimurium* TA98 and TA100 with and without S9 mix.

Sample (mg/plate)	His^+ revertants/plate (% of spontaneous)	
	TA98	TA100
Spontaneous group	42 ± 2 (100)[a]	194 ± 9 (100)[a]
2	40 ± 5 (95)[a]	186 ± 6 (96)[a]
5	41 ± 3 (98)[a]	177 ± 14 (91)[a]
10	35 ± 3 (83)[a]	175 ± 11 (90)[a]
	TA98 + S9	TA100 + S9
Spontaneous group	27 ± 2 (100)[a]	134 ± 5 (100)[a]
2	25 ± 2 (93)[a]	142 ± 8 (106)[a]
5	27 ± 3 (100)[a]	133 ± 7 (99)[a]
10	24 ± 2 (89)[a]	128 ± 9 (96)[a]

Data represent means ± SD for $n = 3$. Values with different superscripts in a column are significantly different ($P < 0.05$). % of spontaneous = ((no. of his^+ revertants in the presence of sample)/(no. of spontaneous revertants)) × 100. The number of spontaneous revertants was determined without samples and mutagens.

Table 6: The antimutagenicity of the aqueous extract of guava twigs (GTE) toward *S. typhimurium* TA98 and TA100.

Sample (mg/plate)	His^+ revertants/plate (% of inhibition)	
	TA98 + 4-NQO	TA100 + 4-NQO
0	456 ± 32 (0)[d]	1848 ± 55 (0)[d]
2	330 ± 13 (30)[c]	1338 ± 54 (31)[c]
5	144 ± 7 (75)[b]	1049 ± 47 (48)[b]
10	97 ± 6 (87)[a]	632 ± 35 (74)[a]
	His^+ revertants/plate (% of inhibition)	
	TA98 + 2-AA + S9	TA100 + 2-AA + S9
0	534 ± 43 (0)[d]	1780 ± 142 (0)[d]
2	270 ± 19 (54)[c]	1326 ± 86 (28)[c]
5	158 ± 14 (74)[b]	717 ± 32 (65)[b]
10	120 ± 11 (82)[a]	491 ± 27 (78)[a]

Data represent means ± SD for $n = 3$. 4-NQO, 4-nitroquinoline N-oxide. 2-AA, 2-anthramine. Values with different superscripts in a column are significantly different ($P < 0.05$). % of inhibition = (1 − ((no. of revertants with mutagen and sample − no. of spontaneous revertants)/(no. of revertants with mutagen − no. of spontaneous revertants))) × 100. The number of spontaneous revertants was determined without samples and mutagen.

GTE on 2-AA induced mutation in *S. typhimurium* TA 98 and TA 100, with S9 activation. GTE at levels of 2–10 mg/plate showed 54–82% inhibition of 2-AA induced mutagenicity in TA 98 and 28–78% inhibition in TA 100. These observations indicated that GTE could inhibit the mutagenicity of both direct and indirect mutagens *in vitro*.

4. Discussion

In this study, GTE demonstrated multiple biological activities, including antimutation, antioxidation, and antityrosinase. A considerable number of studies suggested that the effects of natural antioxidants, such as polyphenols of plant extract, in the biological systems provided protection because they scavenged radicals, chelated metals, and inhibited the oxidases and then regular cellular redox states [17]. Therefore, phenolic constitutes of GTE such as gallic acid, ferulic acid, and myricetin were examined in this study.

As shown in Table 2, GTE and its three marker components exhibited antioxidant activity by scavenging radicals. It was suggested that any antioxidant capacity was due to the development of a reducing ability, when reacting with free radicals, which terminated the radical chain reaction [18]. Additionally, gallic acid showed greater reducing and radical scavenging effects than the other marker constituents did. The levels of total polyphenols and gallic acid in 0.01 mg/mL of GTE were 0.00171 and 0.00011 mg/mL, respectively. As shown in Table 2, total polyphenols of GTE might play a more important role in radical scavenging effect than the three marker compounds did.

Lipid oxidation occurs in cell membranes; it releases arachidonic acid, which is responsible for long-term oxidative stress in cells. In this study, liposome was prepared from phospholipid and used as a lipid oxidation model to imitate the lipid oxidation of biomolecules. GTE demonstrated a protective effect against the lipid damage caused by the hydroxyl radicals produced from a Fenton-like reaction. In fact, 4-hydroxyl-2-nonenal (HNE), a harmful lipid oxidation product, can bind covalently to cellular DNA, to form the exocyclin etheno-DNA-base adduct [19]. In this study, the GTE provided protection against lipid oxidation, indicating that GTE could protect biolipid molecules from oxidative stress and prevent DNA damage in tissues. The levels of total polyphenols, gallic acid, ferulic acid, and myricetin in 0.1 mg/mL of GTE were 0.01710, 0.00111, 0.00091, and 0.00039 mg/mL, respectively. As shown in Table 3, it was possible that the synergistic effects might exist among total polyphenols and the three marker compounds of GTE in liposome protection.

Polyphenols can form complexes with metal ions and exhibit antityrosinase action. Flavonols possessing a 3-hydroxy-4-keto moiety, such as kaempferol or quercetin, inhibited tyrosinase, because of their ability to chelate the copper in the active site of enzyme [20]. This indicated that GTE decreased tyrosinase activity and prevented the progression of browning in processed food. In addition, tyrosinase not only played a critical role in catalyzing the

melanogenesis, but also promoted the reactive metabolites produced during the process of melanin formation. For example, quinonic compounds generated in the polyphenol oxidation process, which then reacted with the free nucleophilic group of proteins, denatured protein structure, and irreversibly destroyed enzyme function [20]. Meanwhile, the synthesis of melanin caused glutathione depletion and the formation of hydrogen peroxide. This study found that GTE decreased tyrosinase activity implying that GTE could prevent a reduction in glutathione and protect cells against quinone induced damage. The equivalent concentrations of total polyphenols, gallic acid, ferulic acid, and myricetin in 0.4 mg/mL of GTE were 0.06840, 0.00444, 0.00364, and 0.00156 mg/mL, respectively. As shown in Table 3, the three marker constitutes of GTE might contribute more tyrosinase inhibition than total polyphenols did.

t-BHP is a well-known strong oxidant which is often used as a reference compound to induce biological injury during different studies. The toxicity of *t*-BHP is related to its induction of mass peroxide production and reduction of intracellular antioxidant levels. As shown in Table 4, GTE could protect liver cells against *t*-BHP-induced oxidative damage. However, the three marker constitutes of GTE displayed an antioxidative role and reduced *t*-BHP-induced cytotoxicity in liver cells. It was also speculate that the reduction of *t*-BHP cytotoxicity by GTE might be attributable to its three phenolic constituents and other active phytochemicals.

Though a number of studies have suggested that some phytochemicals may exhibit mutagenic and cytotoxic activity [21], GTE shows neither toxicity nor mutagenicity toward *S. typhimurium* TA 98 or TA 100 in the present study. The mechanisms of antimutagenesis have different pathways, including deactivation of mutagens, inhibition of metabolic activation of promutagens and deactivation of activated mutagens. In this study, 4-NQO produced the ultimate mutagenic compound, which bound to DNA and generated stable quinolone-purine mono adducts [1]. On the other hand, 2-AA preferred the biotransformation pathway, where a cytochrome P450-dependent monooxygenase produced a nitrene moiety that bound to DNA [2]. Natural products contain many substances that can likely reduce mutation and cancer. For example, *Origanum vulgare* L ssp. *vulgare* [22] and sesame-seed perisperm [23] also show an antimutagenic effect against different mutagens toward *S. typhimurium* mutation. As shown in Table 6, GTE played an antimutagenic role to suppress the mutagenicity of 4-NQO and 2-AA in the Ames test model. These data implied that the conjugated reaction between GTE and the toxic electrophile was an important detoxification pathway. On the other hand, GTE might play an antimutagenic role by scavenging the active metabolic electrophile of 4-NQO and 2-AA. The antimutagenic effects of the GTE might also be attributable to decrease metabolic activation and the levels of toxic reactive intermediates, which further indirectly reduced cellular oxidative stress and, thereby, prevented mutation.

In summary, as mentioned above, GTE demonstrates antimutation, antioxidation, and antityrosinase effects. These activities may be partially attributable to its polyphenolic constituents. Furthermore, the three marker compounds have similar activities to the extract that contain significantly lower levels of the three marker compounds. This suggests that other polyphenols or unknown active components in GTE could also play critical roles in its protective effects. Although these results demonstrate the protective effects provided by GTE against mutagen-induced mutation and *t*-BHP toxicity, further investigations of the nutritional and physiological effects of GTE are still required.

Acknowledgment

This work was supported by the National Science Council of Taiwan, (NSC 99-2313-B-041-001-MY2).

References

[1] Y. Arima, C. Nishigori, T. Takeuchi et al., "4-Nitroquinoline 1-oxide forms 8-hydroxydeoxyguanosine in human fibroblasts through reactive oxygen species," *Toxicological Sciences*, vol. 91, no. 2, pp. 382–392, 2006.

[2] D. G. Baker, H. W. Taylor, S. P. Lee, S. A. Barker, M. E. P. Goad, and J. C. Means, "Hepatic toxicity and recovery of fischer 344 rats following exposure to 2-aminoanthracene by intraperitoneal injection," *Toxicologic Pathology*, vol. 29, no. 3, pp. 328–332, 2001.

[3] W. Takabe, E. Niki, K. Uchida, S. Yamada, K. Satoh, and N. Noguchi, "Oxidative stress promotes the development of transformation: involvement of a potent mutagenic lipid peroxidation product, acrolein," *Carcinogenesis*, vol. 22, no. 6, pp. 935–941, 2001.

[4] H. L. Chu, B. S. Wang, and P. D. Duh, "Effects of selected organo-sulfur compounds on melanin formation," *Journal of Agricultural and Food Chemistry*, vol. 57, no. 15, pp. 7072–7077, 2009.

[5] J. A. O. Ojewole, "Antiinflammatory and analgesic effects of *Psidium guajava* Linn. (Myrtaceae) leaf aqueous extract in rats and mice," *Methods and Findings in Experimental and Clinical Pharmacology*, vol. 28, no. 7, pp. 441–446, 2006.

[6] N. Sambo, S. H. Garba, and H. Timothy, "Effect of the aqueous extract of *Psidium guajava* on erythromycin-induced liver damage in rats," *Nigerian Journal of Physiological Sciences*, vol. 24, no. 2, pp. 171–176, 2009.

[7] J. A. O. Ojewole, "Hypoglycemic and hypotensive effects of *Psidium guajava* Linn. (Myrtaceae) leaf aqueous extract," *Methods and Findings in Experimental and Clinical Pharmacology*, vol. 27, no. 10, pp. 689–695, 2005.

[8] R. M. P. Gutiérrez, S. Mitchell, and R. V. Solis, "*Psidium guajava*: a review of its traditional uses, phytochemistry and pharmacology," *Journal of Ethnopharmacology*, vol. 117, no. 1, pp. 1–27, 2008.

[9] B. S. Wang, P. D. Duh, S. C. Wu, and M. H. Huang, "Effects of the aqueous extract of sugarcane leaves on antimutation and nitric oxide generation," *Food Chemistry*, vol. 124, no. 2, pp. 495–500, 2011.

[10] M. S. Taga, E. E. Miller, and D. E. Pratt, "Chia seeds as a source of natural lipid antioxidants," *Journal of the American Oil Chemists' Society*, vol. 61, no. 5, pp. 928–931, 1984.

[11] B. D. Oomah and G. Mazza, "Flavonoids and antioxidative activities in buckwheat," *Journal of Agricultural and Food Chemistry*, vol. 44, no. 7, pp. 1746–1750, 1996.

[12] M. B. Arnao, A. Cano, and M. Acosta, "The hydrophilic and lipophilic contribution to total antioxidant activity," *Food Chemistry*, vol. 73, no. 2, pp. 239–244, 2001.

[13] M. Oyaizu, "Studies on products of browning reaction: antioxidative activity of products of browning reaction prepared from glucosamine," *Japanese Journal of Nutrition*, vol. 44, pp. 307–315, 1986.

[14] I. Kubo, I. Kinst-Hori, S. K. Chaudhuri, Y. Kubo, Y. Sánchez, and T. Ogura, "Flavonols from Heterotheca inuloides: tyrosinase inhibitory activity and structural criteria," *Bioorganic and Medicinal Chemistry*, vol. 8, no. 7, pp. 1749–1755, 2000.

[15] D. M. Maron and B. N. Ames, "Revised methods for the *Salmonella* mutagenicity test," *Mutation Research*, vol. 113, no. 3-4, pp. 173–215, 1983.

[16] J. L. Mazzei, D. N. da Silva, V. Oliveira et al., "Absence of mutagenicity of acid pyrogallol-containing hair gels," *Food and Chemical Toxicology*, vol. 45, no. 4, pp. 643–648, 2007.

[17] D. Vauzour, A. Rodriguez-Mateos, G. Corona, M. J. Oruna-Concha, and J. P. E. Spencer, "Polyphenols and human health: prevention of disease and mechanisms of action," *Nutrients*, vol. 2, no. 11, pp. 1106–1131, 2010.

[18] D. Huang, O. U. Boxin, and R. L. Prior, "The chemistry behind antioxidant capacity assays," *Journal of Agricultural and Food Chemistry*, vol. 53, no. 6, pp. 1841–1856, 2005.

[19] U. Nair, H. Bartsch, and J. Nair, "Lipid peroxidation-induced DNA damage in cancer-prone inflammatory diseases: a review of published adduct types and levels in humans," *Free Radical Biology and Medicine*, vol. 43, no. 8, pp. 1109–1120, 2007.

[20] Y. J. Kim and H. Uyama, "Tyrosinase inhibitors from natural and synthetic sources: structure, inhibition mechanism and perspective for the future," *Cellular and Molecular Life Sciences*, vol. 62, no. 15, pp. 1707–1723, 2005.

[21] B. N. Ames, "Dietary carcinogens and anticarcinogens: oxygen radicals and degenerative diseases," *Science*, vol. 221, no. 4617, pp. 1256–1263, 1983.

[22] M. Gulluce, M. Karadayi, Z. Guvenalp, H. Ozbek, T. Arasoglu, and O. Baris, "Isolation of some active compounds from *Origanum vulgare* L. ssp. *vulgare* and determination of their genotoxic potentials," *Food Chemistry*, vol. 130, pp. 248–253, 2012.

[23] D. Lazarou, R. Grougnet, and A. Papadopoulos, "Antimutagenic properties of a polyphenol-enriched extract derived from sesame-seed perisperm," *Mutation Research*, vol. 634, no. 1-2, pp. 163–171, 2007.

Hindawi Publishing Corporation
Journal of Chemistry
Volume 2013, Article ID 750623, 6 pages
http://dx.doi.org/10.1155/2013/750623

Research Article

Nutritional and Phytochemical Study of *Ilex paraguariensis* Fruits

Laura Cogoi,[1] M. Silvia Giacomino,[2] Nestor Pellegrino,[2] Claudia Anesini,[1] and Rosana Filip[1]

[1] *Pharmacognosy, Institute of Drug Chemistry and Metabolism (IQUIMEFA), UBA, National Council of Scientific and Technical Research (CONICET), 1113 Buenos Aires, Argentina*
[2] *Bromatology, Faculty of Pharmacy and Biochemistry, University of Buenos Aires, Junín 956, 1113 Buenos Aires, Argentina*

Correspondence should be addressed to Rosana Filip; rfilip@ffyb.uba.ar

Received 26 June 2012; Revised 27 August 2012; Accepted 6 October 2012

Academic Editor: Mehmet Emin Duru

Yerba mate is defined as the product constituted by the dried, slightly roasted, and milled leaves of *Ilex paraguariensis*. However, the fruits of this species are often found in the commercial product. Nowadays the fruits are considered a byproduct. The objective of this work was to obtain the preliminary data of minerals, lipids, methylxanthines and polyphenols in the ripe fruits of *I. paraguariensis*. The results showed a considerable amount of total dietary fiber (42.0 ± 1.6 g/100 g) and nutritionally valuable minerals: potassium (1324 ± 15 mg/100 g), iron (6.4 ± 0.5 mg/100 g), magnesium (168 ± 15 mg/100 g), calcium (150 ± 12 mg/100 g), copper (1.1 ± 0.1 mg/100 g), zinc (2.3 ± 0.3 mg/100 g), and sodium (1.3 ± 0.1 mg/100 g). The lipid content was 4.5 g/100 g. Oleic acid was the predominant unsaturated fatty acid (38.74 ± 0.75 g/100 g). Linoleic acid (1.83 ± 0, 01 g/100 g) was also present. Methylxanthines were quantified: caffeine (0.118 ± 0.001) and theobromine (0.0125 ± 0.0002) g/100 g. The total polyphenol content was 0.717 ± 0.001 g/100 g. The results obtained in this work suggest the potential value of the fruits of *I. paraguariensis* for the development of novel products in the food and pharmaceutical industries. This paper aims to contribute to the scientific knowledge of a natural by-product from industry regarding the need of foods and medicines for the new millennium.

1. Introduction

Ilex paraguariensis St. Hilaire (Aquifoliaceae) is a native tree from Northeastern Argentina, Southern Brazil, and Eastern Paraguay, where it is also cultivated. It is one of the most known and used species in South America since the product obtained from its industrialization, yerba mate, is used to prepare a tea-like beverage (infusions or decoctions) that is appreciated for its peculiar flavour and stimulating, antioxidant, choleretic, and nutritional properties [1, 2]. The leaves are also used in folk medicine to treat arthritis, headache, constipation, rheumatism, obesity, fatigue, fluid retention, and liver disorders. This species is exported to Europe, US, Syria, and Japan where it is marketed as a milled plant or extracts used in herbal formulations and functional food products with stimulating, diuretic, antioxidant, and weight-reducing properties. Yerba mate is included in Codex Alimentarius, Argentine Food Code, Latin-American Food Code, and the main scientific acknowledgment Pharmacopoeias (Martindale, British Herbal Pharmacopoeia, German Commission E Monographs) [3]. Nowadays it is also considered a functional food [4]. According to the Argentine Food Code (CAA), yerba mate is defined as the product constituted exclusively by the dried, slightly roasted, and milled leaves of *I. paraguariensis* which can contain fragments of young branches, pedicles, and floral peduncles. However and as a result of the elaboration process, the fruits of this species may be present in the final product which has been consumed over centuries. The fruit is in a nucule and can reach a diameter of approximately 7 mm; there are four or five single-seed pyrenes (propagules). Mate flowers from October to November and fruiting occurs from December to May. During the ripening process, the fruit color changes from green to white, reddish-brown, and finally black when it is fully ripe. There is a rudimentary embryo in many externally ripe seeds which causes a long period of germination.

The maximum allowed content of fruit or organic material in the final product is 1 g/100 g [5]. The great quantity of fruits remaining after the yerba mate processing is discarded.

Argentina is the main yerba mate producer country, with nearly 280000 tons per year followed by Brazil and Paraguay. The worldwide production of yerba mate has ascended to 874678 tons in 2002 [6]. Nonofficial data suggest that Argentina could generate about 560 tons per year of fruits, and the global annual production could exceed 1700 tons per year. Nowadays the fruits have no economic value and they are treated as waste and used as fertilizer.

The objective of this work was to assess the preliminary data on the nutritional valuable elements, the methylxanthines and total polyphenol content of the ripe fruits of *I. paraguariensis* in order to study their potential value as a source of ingredients to be used in the food and pharmaceutical industries.

2. Materials and methods

2.1. Plant Material. Ripe fruits of *I. paraguariensis* were provided by a "yerba mate" factory located in Gobernador Valentín Virasoro in the province of Corrientes, Argentina. The production process of yerba mate involves the harvest of the green leaves and small stems of this species which contain fruits. They are cut manually, put into 100 kg sacks, and then carried to the factory. After harvesting, they were submitted to a roasting process named "sapecado" (exposition to direct fire at temperatures between 250°C and 550°C during 2–4 min) and then to a drying process (exposition to a current of hot air until a 3-4% of moisture is reached).

The fruits analyzed in this work were harvested in April 2009. They were dark reddish-brown in color, with a diameter of 4 to 6 mm. They were identified by PhD. Gustavo Gibeti, botanical specialist in *Ilex* spp. Comparison with voucher specimens of herbarium standards was done. A sample was deposited in the Herbarium Botany Unit of the Faculty of Pharmacy and Biochemistry of the University of Buenos Aires under number BACP: BAF 2 (series 2010). In our laboratory, the fruits were dried in a stove with hot air circulation and thermostatized at 40°C (until 2% of moisture was reached) and then milled to fine powder with a cutter mill with 1 mm pore mesh.

2.2. Standards and Reagents. Standard solutions CertiPUR Merck Chemicals International were employed for the determination of the mineral content. A mixture of fatty acid methyl esters standards from SUPELCO FAME Mix NHI-C and FAME Mix C4–C24 were purchased from Sigma-Aldrich, Buenos Aires, Argentina. Standards of caffeine and theobromine were purchased from Sigma-Aldrich, Argentina. All solvents and reagents used in the experimental work were analytical-grade chemicals except those used in the high-resolution analytical methods which were HPLC grade and purchased from Merck Chemicals Argentina.

2.3. Proximate Analysis. The recommended methods of the Association of Official Analytical Chemists [8] were adopted to determine the levels of moisture, ash, protein, crude fat, and total dietary fiber. The moisture content was determined by heating 5.0 g of each sample to a constant weight in a crucible placed in an oven maintained at 70°C under pressure (≤50 mm Hg). Ash was determined by calcinations of 3.0 g of each sample placed in muffle furnace maintained at 550°C until constant weight. Protein (% total nitrogen × 6.25) was determined by the Kjeldahl method using 1.0 g samples. Fat content was determined gravimetrically after petroleum ether extraction (boiling point range 35–65°C) of 5.0 g samples.

Total dietary fiber (TDF) was determined by the enzymatic-gravimetric method [9]. The total carbohydrate content was defined as the residue, excluding protein, lipid, TDF, and ash, and was calculated as follows:

$$\text{Total carbohydrate} = 100 - (\%\ \text{moisture} + \%\ \text{ash} + \%\ \text{Protein} + \%\ \text{lipid} + \%\ \text{TDF}). \quad (1)$$

2.4. Mineral Content. Test samples (0.5 g) were digested in 15 mL $HNO_3/HCLO_4$ (2 : 1, v/v) according to AOAC [8], and sodium, potassium, calcium, magnesium, copper, iron, and zinc were determined by atomic absorption spectrophotometry (Perkin–Elmer model AA400).

2.5. Fatty Acids Extraction. Milled fruits (aprox. 9.0 g) were homogenized and lipids were extracted with n-hexane by lixiviation until total extraction [10]. The organic solvent was evaporated under vacuum and the oil obtained was esterified. Methyl esters were prepared by transmethylation according to the procedure of the International Organization for Standardization (ISO) [11] and analyzed by gas chromatography (GC).

2.6. Gas-Liquid Chromatography. The fatty acid methyl esters were analyzed using a Clarus 500 Perkin Elmer gas chromatography device equipped with a flame ionization detector (FID) and the TotalChrom software. A fused silica capillary column SP 2560 (Supelco Park, Bellefonte, PA, USA) (100 m × 0.25 mm and 0.20 μm) was employed. The column temperature was programmed as follows: 150°C (1 min), a gradient ranging from 150°C to 210°C for 20 min at a rate of 5°C/min. The injection port and detector were maintained at 240°C and 280°C, respectively. As carrier gas nitrogen was employed at a gas linear speed of 1.3 mL min^{-1}. The individual fatty acids were identified by comparison of retention times and peak areas with those of known mixtures of fatty acid methyl esters (FAMEs) standards.

2.7. Methylxanthines Extraction. Briefly, 500 mg of each sample were placed in a 250 mL round-bottom flask containing 50 mL of methanol and extracted during 30 minutes under a reflux condenser and then filtered. The residue was subjected to the same procedure twice. The filtrates were then combined and dried in a rotary evaporator [12]. The extract obtained was solubilized in 20 mL of water: acetic acid (98 : 2) and transferred to a 25 mL flask. Methanol was added to reach a

final volume. A 45 μm filter (Millipore) was used to filter the extract before HPLC analysis.

2.8. High Performance Liquid Chromatography. A Varian series 9000 equipment with a Varian 9012 binary pump was used. Quantitation of methylxanthine was done using validated HPLC external standard methods [13]. A reverse-phase IB-SIL RP 18 (5 μm, 250 × 4.6 mm I.D.) Phenomenex column and an elution gradient consisting of solvent A: water : acetic acid (98 : 2) and solvent B: methanol : acetic acid (98 : 2) were used. The elution gradient was: from 17% B to 20% B, 10 min; 20% B (isocratic), 5 min; 20% B to 23% B, 10 min; 23% B to 100% B, 5 min with a flow rate of 1.0 mL·min^{-1}. Identification and quantitation were carried out by simultaneous detection with an UV Varian 9050 UV detector and a Varian 9065 photodiode array detector operating at 273 nm. Samples were injected with a Rheodyne injector fitted with a 100 μL loop.

2.9. Total Polyphenol Determination. The total polyphenol content was determined by spectrophotometry according to the Folin-Ciocalteu method [14] using gallic acid as standard. Exactly around 1.0 mg of methanolic extract was weighted and dissolved in 10 mL of deionized distilled water. Briefly, 1.0 mL of this sample extract was transferred in duplicate to separate tubes containing 7.0 mL distilled water, 0.5 mL of Folin-Ciocalteu's reagent, and 1.5 mL of a 20% sodium carbonate anhydrous solution (added 2 min after the Folin–Ciocalteu's reagent). The tubes were then allowed to stand at room temperature for 60 min and then the absorbance at 765 nm was measured by employing a UV-Vis spectrophotometer (Shimadzu UV 2101). The concentration of polyphenols in samples was derived from a standard curve of gallic acid ranging from 10 to 50 μg/mL (Pearson's correlation coefficient: $r^2 = 0,9996$).

2.10. Statistical Analysis. Data were expressed as means ± standard error of the mean of three independent experiments of the same batch carried out by triplicate.

3. Results and Discussion

3.1. Proximate Analysis. The results of moisture content, ash, lipids, proteins, carbohydrates, and fiber are presented in Table 1. The fruits presented considerable amounts of total dietary fiber (TDF) (42.0 g/100 g) and carbohydrates (38.3 g/100 g).

Since the mid- 1970s, the role of dietary fibers in health and nutrition has received considerable attention [15]. The consumption of dietary and functional fibers has many potential health benefits, namely, the ability to lower the incidence of constipation [16] and irritable bowel syndrome [17], to lower cholesterol levels and diminish the incidence of coronary and cardiovascular heart diseases [18, 19] to prevent obesity [20] and the development of diabetes [21], to avoid colon cancer [22], and to increase survival of patients with breast cancer [23].

Table 1: Proximate composition of the fruits of *Ilex paraguariensis*.

Composition	g/100 g raw fruit, dry weight
Moisture	5.9 ± 0.1
Ash	3.8 ± 0.2
Protein (N× 6.25)	5.5 ± 0.1
Lipid yield	4.5 ± 0.3
Total dietary fiber (TDF)	42.0 ± 1.6
Insoluble dietary fiber (IDF)	37.6 ± 1.3
Soluble dietary fiber (SDF)	4.4 ± 0.3
Carbohydrate	38.3 ± 1.2

Results are expressed as the Means ± SEM of three experiments performed in triplicates. The fiber content has been corrected for protein and ash. The carbohydrate was defined as the residue, excluding protein, lipid, TDF, and ash, and was calculated by difference as follows:
Carbohydrate content = 100 − (% moisture + % ash + % Protein + % lipid + % TDF).

Table 2: Mineral composition of the fruits of *Ilex paraguariensis*.

Element	mg/100 g of dry matter	RDA[(1)]	% RDA[(2)]
Sodium	1.3 ± 0.1	500	0
Potassium	1324 ± 15	2000	66
Iron	6.4 ± 0.5	10	64
Copper	1.1 ± 0.1	0.9	122
Zinc	2.3 ± 0.3	15	14
Calcium	150 ± 12	800	19
Magnesium	168 ± 15	350	48

Results are expressed as the Means ± SEM of three experiments performed in triplicates.
[(1)]RDA (NAS/NRC) [7] based on the recommended daily allowances for adults in the 25–50 age range.
[(2)]% RDA (Mean contribution for mineral requirements in terms of RDA (NAS/NRC) [7].

Dietary fiber-rich products have gained popularity as food ingredients to obtain health benefits and have encouraged food scientists to search for new fiber sources as well as to develop high-fiber products [24].

In the agricultural byproducts of some fruits and greens (apple pomace, citrus fruits, olive cake and oat, among others), TDF content ranges from 10.2 to 87.9 g/100 g [25, 26]. In this work, the insoluble dietary fiber (IDF) (37.6 g/100 g) was the predominant fiber fraction (89.5% of TDF). Similar results were reported for pear pomace (IDF: 82.7% of TDF) and apple pomace (IDF: 77.3% of TDF) [26].

The crude protein content was found to be 5.5 g/100 g and the crude lipids 4.5 g/100 g dry matter. The lipid content is similar to that reported for the aerial parts of legumes, which is about 4-5 g/100 g dry matter [27].

3.2. Mineral Content. The mineral content (sodium, potassium, iron, copper, zinc, calcium, and magnesium) was determined. *I. paraguariensis* fruits contain significant amounts of essential minerals that are associated with improved health status when consumed at doses beyond those necessary for preventing a deficiency state. The results obtained in this work are presented in Table 2. As it is shown, the

Table 3: Fatty acid composition[(1)] of the fruits of *Ilex paraguariensis*.

Fatty acid	%[(1)]
C 6 : 0	2.12 ± 0.16
C 8 : 0	0.68 ± 0.07
C 14 : 0	0.14 ± 0.01
C 16 : 0	30.57 ± 0.79
C 16 : 1	0.38 ± 0.01
C 17 : 0	0.99 ± 0.15
C 18 : 0	12.28 ± 0.19
C 18 : 1 *trans*	0.73 ± 0.23
C 18 : 1	38.74 ± 0.75
C 18 : 1 *cis*	0.65 ± 0.03
C 18 : 2 *trans*	2.21 ± 0.11
C 18 : 2	1.83 ± 0.01
C 20 : 0	0.76 ± 0.01
C 20 : 1	0.24 ± 0.01
C 22 : 0	0.17 ± 0.01
C 24 : 0	0.15 ± 0.03
Total saturated	47.86
Total monounsaturated	40.01
Total polyunsaturated	1.83
Trans fatty acids	2.94
Nonidentified minor components	7.36

[(1)] Percent by weight of total fatty acids identified by GC as fatty acids methyl esters (FAME).
Results are expressed as the means ± SEM of three experiments performed in triplicates.

Table 4: Methylxanthine content in the fruits of *Ilex paraguariensis*.

Methylxanthine	g/100 g raw fruit, dry weight
Caffeine	0.118 ± 0.001
Theobromine	0.0125 ± 0.0002

Data are expressed as the means ± SEM of three independent experiments carried out in triplicates.
The values were obtained by HPLC with DAD. Theophylline was not detected. Detection limit: 1 ppm.

most abundant mineral elements were potassium, iron, and magnesium which represent the 66%, 64%, and 48% of the daily allowances recommended for adults in the 25–50 year age range [7] (Table 2). The high quantity of these elements together with the quantity of calcium and the content of the essential elements zinc and copper allow the fruits to be considered as excellent sources of bioelements [28].

3.3. Fatty Acid Analysis. The yield of fatty acids obtained from the fruits was 4.5 ± 0, 3 g/100 g, where oleic acid as the predominant unsaturated fatty acid reaching a 38.74 g/100 g. Linoleic acid, one of the most important polyunsaturated fatty acids in human food and was also present (1.83 ± 0, 01%). Linoleic acid prevents cardiovascular disorders high blood pressure and is part of the structural components of the plasma membrane [29]. Palmitic and stearic acids were also found at high levels in the lipidic fraction (Table 3).

3.4. Methylxanthine Content. Caffeine and theobromine were identified and quantified. Theophylline was not detected. Results are presented in Table 4. The presence of caffeine in the unripe fruits of this plant has been reported previously and was found to be 0.04 g/100 g [30]. The caffeine and theobromine content (0.118 ± 0.001%) and (0.0125 ± 0.0002) found in this work were higher than those reported by other authors. For example, Schubert et al. [31], who also investigated the unripe fruits, reported an amount of 1.16 ± 0.06 mg/g of total methylxanthines, a value which is also lower than our results. These discrepancies could be due to the fact that our material consisted in the ripe fruits of *I. paraguariensis* and the results previously reported were obtained from the unripe ones. This is the first study on the methylxanthines content in the ripe fruits of *I. paraguariensis*.

3.5. Total Polyphenol Determination. The total polyphenol content found in this work was 0.717 ± 0.001 g/100 g gallic acid equivalents, dry wet. The amount of polyphenols found here was higher than those reported by other authors who studied the unripe fruits. Schubert et al. [31] found $54.25 \pm 1 \times 10^{-3}$ to $110.36 \pm 4 \times 10^{-4}$ 4 mg/g. Borré et al. [30] found 0.03% of chlorogenic acid. These differences could indicate that the ripe fruits contain higher amounts of polyphenols than the unripe ones. This is the first study on the polyphenol content in the ripe fruits of *I. paraguariensis*.

4. Conclusions

This paper aims to contribute to the scientific knowledge of a natural by-product from industry regarding the need of foods and medicines for the new millennium.

The results obtained in this work suggest the potential value of the fruits as a fiber and valuable mineral source. These fruits could also be a source of bioactive compounds such as methylxanthines and polyphenols. The utilization of this material could become profitable and at the same time help to minimize waste disposal problems. The results obtained in this work suggest the potential value of the fruits of *Ilex paraguariensis* for the development of novel products in the food and pharmaceutical industries.

Acknowledgment

This work was supported by Grant UBACYT 20020100-100158 from the University of Buenos Aires.

References

[1] R. Filip, T. Sebastian, G. Ferraro, and C. Anesini, "Effect of *Ilex* extracts and isolated compounds on peroxidase secretion of rat submandibulary glands," *Food and Chemical Toxicology*, vol. 45, no. 4, pp. 649–655, 2007.

[2] S. Gorzalczany, R. Filip, M. D. R. Alonso, J. Miño, G. E. Ferraro, and C. Acevedo, "Choleretic effect and intestinal propulsion of "mate" (*Ilex paraguariensis*) and its substitutes or adulterants,"

Journal of Ethnopharmacology, vol. 75, no. 2-3, pp. 291–294, 2001.

[3] R. Filip and G. E. Ferraro, "Researching on new species of "mate": *Ilex brevicuspis*: phytochemical and pharmacology study," *European Journal of Nutrition*, vol. 42, no. 1, pp. 50–54, 2003.

[4] S. Isolabella, L. Cogoi, P. López, C. Anesini, G. Ferraro, and R. Filip, "Study of the bioactive compounds variation during yerba mate (*Ilex paraguariensis*) processing," *Food Chemistry*, vol. 122, no. 3, pp. 695–699, 2010.

[5] CAA, "Código Alimentario Argentino," (Argentine Food Code), art. 1193-1198, 2000, http://www.anmat.gov.ar/ alimentos.

[6] C. I. Heck and E. G. de Mejia, "Yerba mate tea (*Ilex paraguariensis*): a comprehensive review on chemistry, health implications, and technological considerations," *Journal of Food Science*, vol. 72, no. 9, pp. R138–R151, 2007.

[7] RDA and NAS/NRC, *Recommended Dietary Allowances*, National Academy of Sciences/National Research Council, Washington, DC, USA, 10th edition, 1989.

[8] Association of Official Analytical Chemists, *Official Methods of Analysis*, Association of Official Analytical Chemists, Washington, DC, USA, 17th edition, 2000.

[9] L. Prosky, N. G. Asp, T. F. Schweizer, J. W. DeVries, and I. Furda, "Determination of insoluble, soluble, and total dietary fiber in foods and food products: interlaboratory study," *Journal of the Association of Official Analytical Chemists*, vol. 71, no. 5, pp. 1017–1023, 1988.

[10] M. F. Ramadan, G. Sharanabasappa, S. Parmjyothi, M. Seshagiri, and J. T. Moersel, "Profile and levels of fatty acids and bioactive constituents in mahua butter from fruit-seeds of buttercup tree [*Madhuca longifolia* (Koenig)]," *European Food Research and Technology*, vol. 222, no. 5-6, pp. 710–718, 2006.

[11] ISO, "International organization for standardization: animal and vegetable fats and oils," in *Preparation of Methyl Esters of Fatty Acids, ISO, 5509*, pp. 01–06, 1978.

[12] *Real Farmacopea Española*, Monografía Nuez de cola, 3rd edition, 2005.

[13] R. Filip, P. López, J. Coussio, and G. Ferraro, "Mate substitutes or adulterants: study of xanthine content," *Phytotherapy Research*, vol. 12, no. 2, pp. 129–131, 1998.

[14] V. L. Singleton and J. A. Rossi, "Colorimetry of total phenolics with phosphomolybdic phosphotungstic acid reagents," *American Journal of Enology and Viticulture*, vol. 16, no. 3, pp. 144–158, 1965.

[15] A. Abdul-Hamid and Y. S. Luan, "Functional properties of dietary fibre prepared from defatted rice bran," *Food Chemistry*, vol. 68, no. 1, pp. 15–19, 2000.

[16] G. Castillejo, M. Bulló, A. Anguera, J. Escribano, and J. Salas-Salvadó, "A controlled, randomized, double-blind trial to evaluate the effect of a supplement of cocoa husk that is rich in dietary fiber on colonic transit in constipated pediatric patients," *Pediatrics*, vol. 118, no. 3, pp. e641–e648, 2006.

[17] S. Malhotra, S. V. Rana, S. K. Sinha, and S. Khurana, "Dietary fiber assessment of patients with irritable bowel syndrome from northern India," *Indian Journal of Gastroenterology*, vol. 23, no. 6, pp. 217–218, 2004.

[18] A. L. Romero, K. L. West, T. Zern, and M. L. Fernandez, "The seeds from Plantago ovata lower plasma lipids by altering hepatic and bile acid metabolism in guinea pigs," *Journal of Nutrition*, vol. 132, no. 6, pp. 1194–1198, 2002.

[19] G. M. A. Van Rosendaal, E. A. Shaffer, A. L. Edwards, and R. Brant, "Effect of time of administration on cholesterol-lowering by psyllium: a randomized cross-over study in normocholesterolemic or slightly hypercholesterolemic subjects," *Nutrition Journal*, vol. 3, article 17, 2004.

[20] K. Murakami, S. Sasaki, H. Okubo, Y. Takahashi, Y. Hosoi, and M. Itabashi, "Dietary fiber intake, dietary glycemic index and load, and body mass index: a cross-sectional study of 3931 Japanese women aged 18–20 years," *European Journal of Clinical Nutrition*, vol. 61, no. 8, pp. 986–995, 2007.

[21] J. M. A. Hannan, L. Ali, B. Rokeya et al., "Soluble dietary fibre fraction of *Trigonella foenum-graecum* (fenugreek) seed improves glucose homeostasis in animal models of type 1 and type 2 diabetes by delaying carbohydrate digestion and absorption, and enhancing insulin action," *British Journal of Nutrition*, vol. 97, no. 3, pp. 514–521, 2007.

[22] K. Wakai, C. Date, M. Fukui et al., "Dietary fiber and risk of colorectal cancer in the Japan collaborative cohort study," *Cancer Epidemiology Biomarkers and Prevention*, vol. 16, no. 4, pp. 668–675, 2007.

[23] A. J. McEligot, J. Largent, A. Ziogas, D. Peel, and H. Anton-Culver, "Dietary fat, fiber, vegetable, and micronutrients are associated with overall survival in postmenopausal women diagnosed with breast cancer," *Nutrition and Cancer*, vol. 55, no. 2, pp. 132–140, 2006.

[24] G. Schaafsma, "Health claims, options for dietary fiber," in *Dietary Fiber*, J. M. van der Kamp, N. G. Asp, J. Miller, and G. Schaafsma, Eds., pp. 27–38, Academic Publishers, Wageningen, the Netherlands, 2004.

[25] N. Grigelmo-Miguel and O. Martín-Belloso, "Characterization of dietary fiber from orange juice extraction," *Food Research International*, vol. 31, no. 5, pp. 355–361, 1998.

[26] G. Laufenberg, B. Kunz, and M. Nystroem, "Transformation of vegetable waste into value added products: (A) the upgrading concept; (B) practical implementations," *Bioresource Technology*, vol. 87, no. 2, pp. 167–198, 2003.

[27] I. A. Ajayi, R. A. Oderinde, D. O. Kajogbola, and J. I. Uponi, "Oil content and fatty acid composition of some underutilized legumes from Nigeria," *Food Chemistry*, vol. 99, no. 1, pp. 115–120, 2006.

[28] F. Saura-Calixto and J. Cañellas, "Mineral composition of almond varieties (*Prunus amygdalus*)," *Zeitschrift für Lebensmittel-Untersuchung und-Forschung*, vol. 174, no. 2, pp. 129–131, 1982.

[29] A. A. Omode, O. S. Fatoki, and K. A. Olaogun, "Physicochemical properties of some underexploited and nonconventional oilseeds," *Journal of Agricultural and Food Chemistry*, vol. 43, no. 11, pp. 2850–2853, 1995.

[30] G. L. Borré, S. Kaiser, C. Pavei, F. A. da Silva, V. L. Bassani, and G. G. Ortega, "Comparison of methylxanthine, phenolics and saponin contents in leaves, branches and unripe fruits from *Ilex paraguariensis* A. St. Hil. (mate)," *Journal of Liquid Chromatography and Related Technologies*, vol. 33, no. 3, pp. 362–374, 2010.

[31] A. Schubert, D. F. Pereira, F. F. Zanin, S. H. Alves, R. C. R. Beck, and M. L. Athayde, "Comparison of antioxidant activities

and total polyphenolic and methylxanthine contents between the unripe fruit and leaves of *Ilex paraguariensis* A. St. Hil," *Pharmazie*, vol. 62, no. 11, pp. 876–880, 2007.