J Wood Sci (2013) 59:426–431
DOI 10.1007/s10086-013-1344-4

ORIGINAL ARTICLE

Anti-acne activity of tannin-related compounds isolated from *Terminalia laxiflora*

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Received: 21 February 2013 / Accepted: 17 April 2013 / Published online: 17 May 2013

Abstract In our investigation to find out new anti-acne agent, we focused on *Terminalia laxiflora* Engl & Diels (Combretaceae) methanolic wood extract, which has been selected during previous screening experiments for anti-acne agents, which included 29 species of Sudanese medicinal plants. Based on the biologically guided fractionation using an antibacterial assay against *Propionibacterium acnes*, a lipase inhibitory assay and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity assay; five tannin-related compounds were isolated, such as ellagic acid, flavogallonic acid dilactone, terchebulin and gallic acid. Terchebulin showed good antibacterial activity; minimum inhibitory concentration (MIC) = 125 μg/ml and minimum bactericidal concentration (MBC) = 250 μg/ml. Gallic acid exhibited lipase inhibitory activity with IC_{50} value of 149.3 μM, which showed strong inhibition compared with terchebulin, IC_{50} 260.7 μM. However, all compounds exhibited better or equal DPPH radical scavenging activity to (+)-catechin as positive control. Ellagic acid and terchebulin showed the best DPPH radical scavenging activities, IC_{50} 4.86 and 4.90 μM, respectively. This study demonstrated that terchebulin has potentiality as an anti-acne agent.

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Keywords *Terminalia laxiflora* Engl & Diels · *Propionibacterium acnes* · Lipase inhibitor · Terchebulin · Flavogallonic acid dilactone

Introduction

Acne vulgaris is the most common skin disease that affects areas containing the largest oil glands including face, back and trunk [1]. *Propionibacterium acnes* have been implicated over other cutaneous microflora in contributing to the inflammatory response of acne. It acts as an immunostimulator through producing enzymes such as lipases and proteases which are involved in the development of inflammatory process [2]. Several treatments have been introduced to decrease the esthetic and psychological problems caused by acne. The topical application of therapeutic agents has been found to be more feasible than hormonal treatment and laser therapy. The ingredients in topical acne treatments, particularly herbs and naturally derived compounds, have received considerable interest as they show fewer adverse effects than synthetic agents [3]. Based on our previous screening experiments for anti-acne activity, the methanolic and 50 % ethanolic extracts of *Terminalia laxiflora* Engl & Diels wood showed potent activity among the 29 species of Sudanese medicinal plants examined [4].

The genus *Terminalia* is the second largest genus of Combretaceae family consisting of 200 species, distributed in the tropics and subtropics. About 30 species of *Terminalia* are found in Africa [5]. About 200 woody species of *Terminalia* are used as resources in the timber, pharmaceutical, and leather industries [6]. Although traditional healers throughout Africa have used species of the Combretaceae for the treatment of a wide range of disorders,

only a few species have been subjected to scientific studies [7]. Several biological activities of *Terminalia* sp. have been reported such antibacterial, antifungal [8], anticancer [9], anti-inflammatory [10], antidiabetic [11], hypocholesterolemic [12], anti-ulcer activity [13], anticaries agent [14], anti-HIV-1, anti-malarial [15], antioxidant and melanin inhibitory activity [16]. These pharmacological studies in a number of *Terminalia* species have led to isolation of several compounds such as gallic acid, ellagic acid, punicalagin, terchebulin, isoterchebulin, chebulagic acid, chebulinic acid and others.

T. laxiflora is a common indigenous tree species in woodland and semi-humid Savannah of the Sudan. It has multipurpose uses with a high potential of timber production and traditional medicinal uses. In Sudan decoction of *T. laxiflora* stem bark is used for malaria and cough treatments [17]. Women also use heartwood for fumigant "smoke bath". Exposure to the smoke bath is believed to relieve rheumatic pain, smoothen skin and achieve general body relaxation besides other cosmetic and medicinal beautification [18]. Earlier work done on the root bark of *T. laxiflora* has led to isolation of several compounds like laxiflorin, ellagic acid, trimethylellagic acid, tetramethylellagic acid and terminolic acid [19].

In this study five compounds from methanolic wood extract of *T. laxiflora* have been isolated and evaluated for their anti-acne activities using antibacterial assay against *P. acnes*, a lipase inhibitory assay and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity assay.

Materials and methods

Plant materials

T. laxiflora wood was purchased from Khartoum, Sudan, in March 2011. The specimen was authenticated by Dr. Ashraf Mohamed (Faculty of Forest, University of Khartoum). Voucher specimen (SD-KH-03) was deposited at the Department of Horticulture, Faculty of Agriculture, University of Khartoum.

Extraction and isolation

T. laxiflora wood (300 g) was air-dried at room temperature, powdered and extracted with 3 L of methanol for 12 h three times. The extract was filtered then the solvent was removed under vacuum using rotary evaporator at 30 °C. The yield of methanol extract was 42.9 g (14.3 %).

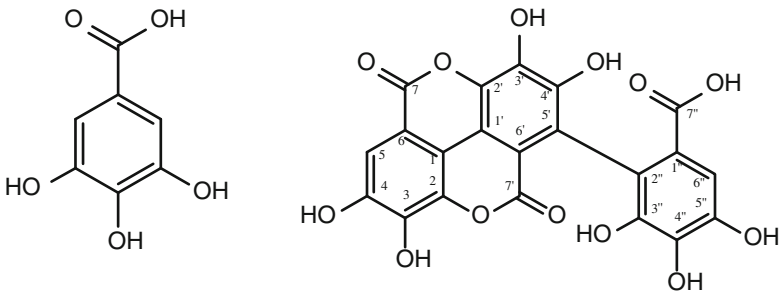
Part of the extract (10 g) was separated by medium pressure liquid chromatography (MPLC) using ODS column (YMC-DispoPack AT ODS-25:120 g).The column was conditioned with the first eluent used for separation for 30 min with flow rate 0.5 ml/min to ensure that no other contaminations were present on the column. MPLC separation was performed using a chromatography pump (540 Yamazen, Japan), UV detector at 280 nm wavelength (UV-10V Yamazen, Japan) and fraction collector (SF-2120, Advantec Toyo Ltd., Japan).

Elution with H_2O/MeOH = 95/5 (v/v) and 20/80 (v/v) gave two fractions (F_1 and F_2). Fraction one (F_1) (200 mg) was subjected to column chromatography on a Sephadex LH-20 using MeOH and 70 % acetone in water (v/v) as the eluent to give four fractions (F1-1–F1-4). Separation of these sub-fractions (F1-1, F1-2 and F1-4) were performed using preparative high performance liquid chromatography (HPLC) with reversed phase Inertsil OD-3 column (GL Sciences Inc. 10 mm i.d. × 250 mm) monitored at 280 nm. The solvent system used was as follows: a gradient program for 60 min from 10 to 100 % methanol in water with 0.05 % TFA at a flow rate 5 ml/min. This separation gave gallic acid (**1**) 10 mg, flavogallonic acid dilactone (**2**) 27.5 mg and terchebulin (**3**) 16.9 mg. Fraction two (F_2) was also subjected to preparative HPLC under the same condition to isolate ellagic acid (**4**) 35 mg. Chemical structures of the isolated compounds (**1**)–(**4**) are shown in Fig. 1.

Identification of isolated compounds from *T. laxiflora*

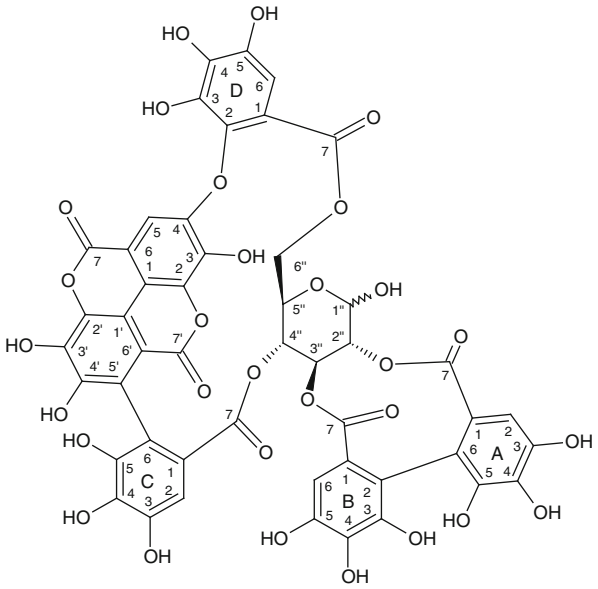
Compounds were identified by ^{1}H, ^{13}C nuclear magnetic resonance (NMR) and liquid chromatography–mass spectrometry (LC–MS). Methanol-d_4 was used as the NMR solvent. NMR measurements were obtained by using JEOL ECP 600 MHz NMR. LC–MS (Waters Waters®XevoTM QTof MS) measurements were performed using column C_{18} (2.1 mm i.d. × 100 mm) with MeOH/H_2O = 5/95 (v/v) (30 min), 100/0 (10 min) with a linear gradient as eluent. The data were collected in negative ionization mode. Spectroscopic data of flavogallonic acid dilactone (**2**) and terchebulin (**3**) are known from the literature. Gallic acid (**1**) and ellagic acid (**4**) were identified by comparing the spectroscopic data of commercial reagents.

Compound **2** Flavogallonic acid dilactone, a tan powder. LC–MS (negative ion mode) *m/z*: 469(M-H); ^{1}H-NMR (in CD_3OD): δ (ppm) 7.26 (s), 7.50 (s). ^{13}C-NMR (in CD_3OD): δ (ppm) 108.1 (C-1, 1′), 110.1–114.4 (C-6, 6′), 112.8 (C-5), 113.3 (C-6″), 117.5–120.2 (C-5′, 2″), 124.9 (C-1″), 135.7 (C-2), 136.3 (C-3), 136.5 (C-4), 137.8 (C-2′), 139.2 (C-3′), 143.2 (C-4′), 144.1 (C-3″), 145.9 (C-4″), 147.8 (C-5″), 158.9-160.4 (C-7, 7′), 168.9(C-7″).

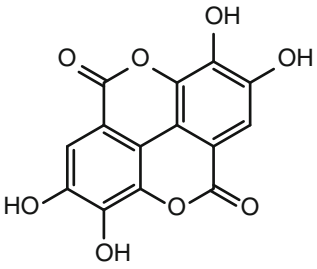


Compound (**1**)

Compound (**2**)



Compound (**3**)



Compound (**4**)

Fig. 1 Structures of compound (**1**) gallic acid, compound (**2**) flavogallonic acid, Compound (**3**) terchebulin and compound (**4**) ellagic acid

^{1}H-NMR and ^{13}C-NMR spectral data coincided to that of published report [20].

Compound **3** Terchebulin, Tan powder. LC–MS (negative ion mode) *m/z*: 1,083 (MH); ^{1}H-NMR (in CD_3OD): δ (ppm) 3.04 (t, $J = 11.6$ Hz, one of the H-6″), 4.21 (t, $J = 10.3$ Hz, H-5), 4.48 (t, $J = 8.9$ Hz, one of the H-6″), 4.78 (t, $J = 11.0$ Hz, H-4″), 4.98 (dd, $J = 3.5$, 9.7 Hz, H-2″), 5.23 (d, $J = 2.8$ Hz, H-1″), 5.64 (t, $J = 9.6$ Hz, H-3″), 6.37 (s, H-B6), 6.42 (s, H-D6), 6.56 (s, H-A2), 6.79 (s, H-C2), 7.48 (s, H-5). ^{13}C-NMR (in CD_3OD): δ (ppm) 63.4 (C-6″), 68.5 (C-4″), 69.0 (C-5″), 74.1 (C-3″), 74.2 (C-2″), 90.2 (C-1″), 106.4 (C-B6), 106.5 (C-D6), 106.8 (C-A2), 108.5(C-C2), 112.0-114.0 (C-A6, B2, 5, 5′, 1, 1′, 2, 2′, 6, 6′), 116.0 (C-C6), 122.2 (C-D1), 123.5 (C-B1,C1), 125.1 (C-A1), 135.9 (C-B4), 136.1 (C-A4), 137.5 (C-C4), 137.6 (C-D4), 138.4 (C-3), 139.1 (C-D3), 140.7 (C-3′), 141.7 (C-D2), 143.4-143.6 (C-A5, B3, C5), 144.5-144.6 (C-A3, B5, C3, D5), 147.4 (C-4′), 150.3 (C-4), 158.3 (C-7′), 159.5 (C-7), 166.9 (C-D7), 167.0 (C-C7), 168.9 (C-A7), 169.5 (C-B7).

^{1}H-NMR and ^{13}C-NMR spectral data coincided to that of published report [21].

Bioassay tests

Evaluation of antibacterial activity against Propionibacterium acnes ATCC 6919

The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values were determined by broth dilution assay which was performed by the method of Batubara et al. [22]. In brief 100 µl of each compound were twofold serially diluted with dimethyl sulfoxide (DMSO) 10 % in water (v/v), 95 µl of sterilized medium and inoculum 5 µl were added to each well of a 96-well plate. The inoculum was prepared at the density of 1×10^6 CFU/ml approximately. The broth culture was incubated for 72 h under anaerobic conditions. Compound concentrations at which there was no visually detectable bacterial growth was described as the MIC. Next 10 µl of each medium with no visually detectable bacterial growth was inoculated in 100 µl of fresh medium. The concentration at which there was no bacterial growth after the second inoculation was described as the MBC.

The bioassay tests including lipase inhibitory activity assay and DPPH radical scavenging activity assay were performed as previously described by Muddathir et al. [4].

Results and discussion

P. acnes, an anaerobic pathogenic inhabitant of human skin, plays an important role in pathogenesis of acne. For many years antibiotics and hormones were usually applied to treat acne [23, 24]. However, these agents are often accompanied by severe side effects and drug resistance [25, 26]. Therefore, many researchers have tried to develop therapeutic agents for acne that have less side effects and high antibacterial activity.

Antibacterial activities of the compounds isolated from *T. laxiflora* towards *P. acnes* were analyzed. In Table 1 MIC and MBC data indicated that terchebulin exhibited the strongest inhibitory activities (MIC = 125 μg/ml and MBC = 250 μg/ml). Silva et al. [27] reported that the ellagitannins terchebulin and punicalagin isolated from *T. macroptera* root had anti-*Helicobacter pylori* activity with MIC value 200 μg/ml. In addition, chebulagic acid isolated from the fruit of *T. chebula* showed inhibitory activity against *P. acnes* with MIC value of 12.5 μg/ml [28]. On the other hand, ellagic acid showed good MIC value (125 μg/ml) with low MBC value (1,000 μg/ml). According to Panichayupakaranant [29] ellagic acid-rich pomegranate rind extracts exhibited potent bacteriostatic effect against *P. acnes* with an MIC value of 15.6 μg/ml. The antibacterial activities of these four compounds were lower than tetracycline. However, the effectiveness of terchebulin, ellagic acid and flavogallonic acid dilactone are better than isopropyl methylphenol (IPMP) as positive control. Gallic acid showed low MIC and MBC value of 2,000 μg/ml. These values are consistent with the result previously reported [28].

Earlier studies have suggested that the fatty acids produced by lipase activity from *P. acnes* resulted in inflammation owing to neutrophil chemotaxis [30]. A decline in free fatty acids caused by lipase inhibition is associated with a decrease in the growth of *P. acnes* [31]. Natural inhibitors of lipase such as flavonoids were reported as promising candidates for acne treatment [32]. In addition, therapeutic agents for acne-like antibiotics are usually employed to inhibit inflammation or kill the bacteria. Several reports also suggest that in case of tetracycline, erythromycin and clindamycin, several side effects were observed such as appearance of resistant bacteria, organ damage and immunohypersensitivity if they are taken for a long time [33]. In the present study, lipase inhibitory activity of isolated compounds was also evaluated. The results in Table 1 revealed that gallic acid and terchebulin have anti-lipase activity with IC_{50} value of 149.3 and 260.7 μM, respectively. Previously, inhibitory activities of kaempferol and glycyrrhizic acid on *P. acnes* lipase has been reported and their IC_{50} (240–340 μM) were evaluated as being fairly high lipase inhibitors [32]. In comparison with the above, this study has indicated that inhibitory level of terchebulin is less than gallic acid; however, it is still in the range of natural inhibitor of lipase. These results are in contradiction to that of Patil et al. [28] who stated that gallic acid did not inhibit lipase activity of *P. acnes* (with IC_{50}>5,000 μM); furthermore, he mentioned that *T. chebula* fruit extract showed significant inhibition of lipase activity (IC_{50} = 60 μM). Various studies support our results that gallic acid possess anti-lipase activity but specifically for pancreatic lipase [34]. Makihara et al. [35] concluded that gallic acid is the active ingredient of *T. bellirica* fruits that causes pancreatic lipase inhibition. Although several enzymatic activities are affected by ellagitannin-related compounds and could be influenced by the number and orientation of the galloyl group [20], it was a surprise for us to report that gallic acid alone could have high activity. This inhibition of lipase by this compound might be due to the binding of the substrate or the interaction with enzyme. Further research is needed to determine the mechanism of inhibition induced by these

Table 1 Anti-*Propionibacterium acnes*, lipase inhibitory and antioxidant activities of isolated compounds from *T. laxiflora*

Sample	MIC (μg/ml)	MBC (μg/ml)	Lipase inhibition IC_{50} (μM)	Antioxidant IC_{50}(μM)
Gallic acid	2,000	2,000	149.31 ± 10.19	8.90 ± 0.30
Ellagic acid	125	1,000	832.59 ± 7.09	4.86 ± 0.03
Flavogallonic acid dilactone	250	1,000	534.80 ± 6.99	7.03 ± 0.43
Terchebulin	125	250	260.65 ± 1.60	4.90 ± 0.10
Tetracycline	0.03	0.122	883.30 ± 4.94	nd
IPMP	1,000	2,000	>3,328.45	nd
(+)-Catechin	nd	nd	>1,722.53	8.23 ± 0.12

MIC minimal inhibitory concentration, *MBC* minimal bactericidal concentration, *IC_{50}* 50 % inhibitory concentration, *IPMP* 3-methyl 4-isopropylphenol, *nd* not determined

compounds. The IC_{50} values of flavogallonic acid dilactone and ellagic acid were (534.80 and 832.59 μM, respectively). Although tetracycline is a potent antimicrobial agent, it has inhibitory activity against *P. acnes* lipase and is used as positive control [22, 28]. Tetracycline has low lipase inhibitory activity (883.3 μM) as compared to gallic acid, terchebulin and flavogallonic acid dilactone. Previously Batubara et al. [22] also reported that brazilin isolated from *Caesalpinia sappan* L. wood had strong lipase inhibition than tetracycline. To our knowledge, this is the first time that the anti-lipase activity of terchebulin and flavogallonic acid dilactone has been investigated.

Reactive oxygen species (ROS) including singlet oxygen, superoxide anion, hydrogen peroxide, lipid peroxide and nitric oxide play an important role in inflammatory acnes as well as tissue injury. ROS promote tumor necrosis factor formation [36] and consequently activate T lymphocytes and keratinocytes. Also the cytokines and other proinflammatory compounds are produced and released causing microcomedones. These microcomedones further develop into comedones and inflammatory lesions [3]. ROS are subsequently generated from the hyper-colonization of *P. acnes* and from ultraviolet exposure [37]. Chemotactic substances released from the bacteria attract polymorphonuclear leukocytes to the site of inflammation [38]. After phagocytosis of the bacteria, the attracted neutrophils are thought to release lysosomal enzymes and produce (ROS) that can damage the follicular epithelium. Besides that stimulation of epidermal cells by *P. acnes* leads to generation of ROS, particularly superoxide anion. This phenomenon is associated with production of a soluble proinflammatory molecule and epidermal cell death. [39].

Therefore, compounds that inhibit the growth of skin microorganisms and have antioxidant activity are required. We examined the isolated compounds for their antioxidant activities using DPPH radical scavenging assay as shown in Table 1, Ellagic acid and terchebulin exhibited strong antioxidant activity with IC_{50} 4.86 M and 4.90 μM, respectively, when compared to positive control of (+)-catechin (IC_{50} 8.23 μM). However, flavogallonic acid dilactone and gallic acid showed relatively similar activity to (+)-catechin (IC_{50} 7.03 and 8.90 μM, respectively). Yokozawa et al. [40] reported that most of the tannins with low concentration have high activity than flavonoids on DPPH radical scavenging. An increase in galloyl group, molecular weight and ortho-hydroxyl structure enhanced the antioxidant activity of tannins. Antioxidant activity using DPPH radical scavenging, oxygen radical absorbance capacity (ORAC) and ferric reducing ability of plasma (FRAP) in vitro assays indicated that chebulic ellagitannins have high antioxidant activity within isolated compounds from three *Terminalia* sp. [41]. Manosroi et al. [16] showed that isoterchebulin and other hydrolyzable tannins isolated from galls of *T. chebula* had strong radical scavenging activity.

From the data presented in this study, it is evident that some isolated compounds such as ellagic acid, gallic acid and flavogallonic acid dilactone from the methanolic extracts of *T. laxiflora* wood have potentiality as anti-acne agent. Terchebulin showed excellent potency as anti-acne agent, since it demonstrated antibacterial activity against *P. acnes* (MIC = 125 μg/ml MBC = 250 μg/ml) with strong antioxidant activity and good lipase inhibitory activity.

Acknowledgments The authors thank Dr. Ashraf Mohamed from the Faculty of Forestry, University of Khartoum, for his assistance in the plants identification and authentications. This project was supported financially by the Ministry of Education, Culture, Sports, Science and Technology; Japan (MEXT Grant).

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