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GAVIN PUBLISHERS

Journal of Oncology Research and Therapy

Ahmed M, et al. J Oncol Res Ther: JONT-167.

DOI: 10.29011/2574-710X. 000067

Research Article

Isolation of Two Penta-Cyclic Triterpenoids from *Quercus Dilatata* L. and Evaluation of Their Chemo-Preventive Potential

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Citation: Ahmed M, Kondratyuk TP, Pezzuto JM, Groundwater PW, Ul-Haq I (2018) Isolation of Two Penta-Cyclic Triterpenoids from *Quercus Dilatata* L. and Evaluation of Their Chemo-Preventive Potential. J Oncol Res Ther: JONT-167. DOI: 10.29011/2574-710X. 000067

Received Date: 26 September, 2018; **Accepted Date:** 10 October, 2018; **Published Date:** 18 October, 2018

Abstract

Natural products especially medicinal plants have served as reserve of bioactive secondary metabolites which are in continuous phase of isolation and identification for therapeutic purpose. Present study was designed to explore the phytochemical riches of *Quercus dilatata* L. Two pentacyclic triterpenoids named as friedelin and epifriedelinol were isolated from n-hexane fraction of crude methanolic extract of *Quercus dilatata* aerial parts by bioassay guided separation techniques. Normal phase silica gel column chromatography was used for the separation of compounds. The structures were mainly characterized by means of nuclear magnetic resonance and electron ionization mass spectrometry, after comparing with the data from literature. These compounds have been isolated for the first time from *Q. dilatata*. Among the two triterpenoids, friedelin showed substantial inhibition of nitric oxide production in lipopolysaccharide-activated murine macrophage raw 264.7 cells (63.0 ± 5.3%). It also significantly inhibited tumor necrosis factor-α activated nuclear factor-κB in stable transfected human embryonic kidney cells 293 (65.2 ± 3.2%). Survival rates of cells in both assays were 92.3 ± 2.3% and 100 ± 8.4% respectively. Further it also showed moderate protein kinase inhibitory activity against mutated *Streptomyces* 85E strain (7.0 ± 0.5 bald zone of inhibition) in hyphae formation inhibition assay. These results suggest the possible applications of friedelin as a good chemo-preventive agent.

Introduction

Cancer is considered as a leading anomaly, causing an outburst of mortalities worldwide. An estimated 14.1 million new cases and 8.2 million deaths had occurred in 2012, especially in developing countries owing to inadequate healthcare facilities. Mostly lungs and breast cancers are the main causes of deaths in men and women respectively [1]. Current therapeutic strategies equipped with targeted drug delivery systems have undoubtedly reduced the sufferings of cancer patients. However; prevention, blockade or reversal of this disease is foremost concern; making the search for newer and safer chemo-preventive agents inevitable. Natural products particularly medicinal plants have served as an incessant source of medicines since antiquity. Various secondary metabolites have been isolated and gained profound attention including several anticancer agents such as vincristine, vinblastine, taxol, camptothecin etc. The quest for identification, isolation and characterization of novel anticancer and chemo-preventive agents from plants is in continuity, which prompted us to explore a previously untapped medicinal plant; *Quercus dilatata*.

Quercus dilatata Lindl. ex Royle (Synonym; *Quercus floribunda* Lindl. Camus), commonly known as Holly Oak and locally as Bunj or Barungi, belongs to family Fagaceae. The genus *Quercus* is comprised of around 500 species, some of them previously reported to possess antioxidant [2], antibacterial [3-5] and gastroprotective effects [6]. The seeds and leaves of *Quercus dilatata* are traditionally used for antidiarrheal, diuretic and astringent effects [7] as well as for the eradication of urinary tract infections, gonorrhea, sore throat and mouth [8,9]. Due to its medicinal importance, this plant was selected for the isolation of bioactive compounds. In the present study, we report the isolation of epifriedelinol and friedelin from aerial parts of *Q. dilatata*. These two pentacyclic triterpenoids have been isolated from various species [10-12] and reported to possess antibacterial, antifungal, anti-inflammatory, antioxidant and cytotoxic activities

[13,14], but there is need to identify underlying mechanisms and to ascertain their chemo-preventive perspective. To the best of our knowledge, isolation of friedelin and epifriedelinol from *Q. dilatata* and determination of their protein kinase inhibitory potential in *Streptomyces* 85E strain have been reported for the first time in the current study.

Materials and Methods

Plant Collection

Aerial parts (stems and leaves) of *Q. dilatata* were collected during September 2013 from Murree hills, Pakistan and identified by Prof. Dr. Rizwana Aleem Qureshi, Department of Plant Sciences, Faculty of Biological Sciences, Quaid-i-Azam University Islamabad, Pakistan. Voucher specimen (PHM-490) was deposited in the herbarium of medicinal plants, Department of Pharmacy, Quaid-i-Azam University, Islamabad.

Extraction, Isolation and Identification of Compounds

The dried and crushed plant material (10 kg) was extracted with chloroform: methanol (CH_3Cl:CH_3OH, 1:1, 40 L) at room temperature. Solvent was then filtered, concentrated in rotary evaporator (Buchi, Switzerland) and dried in vacuum oven (Yamato, Japan) at 45˚C to obtain crude extract (1200 g). The crude extract was then suspended in distilled water (4 L) and extracted with n-hexane (NH, 3 × 4 L), ethyl acetate (EA, 3 × 4 L), and n-butanol (NB, 3×4 L) successively. All the fractions were subjected to cancer chemopreventive mechanistic bioassays and based upon the results; NH fraction was selected for isolation. NH fraction (760 g) was subjected to silica gel column chromatography (CC, ø 15 cm, silica gel 60, 70-230 mesh, Merck, Germany, 3 kg) using a gradient of NH-EA (1:0 to 0:1), to yield 54 fractions. These fractions were subjected to analytical normal phase thin layer chromatography (silica gel 60 F254, Merck, Germany) and those having similar R_f were pooled together to yield 13 master fractions (QDNA-QDNM). Fraction QDNA (60.21 g) was then subjected to silica gel CC (ø 15 cm, 70-230 mesh, 500 g), using NH: EA (1:0 to 0:1) as mobile phase, yielding 32 subfractions (QDNA1 to QDNA32, 50 ml each). QDNA27 and QDNA30 subfractions were then evaporated individually and washed with NH: EA (10:1) to yield colorless crystals.

The compounds were identified by spectral studies such as ^1H and ^{13}C Nuclear Magnetic Resonance (NMR) and EIMS. Both ^1H and ^{13}C spectra were recorded using Varion 400 MR with a SMS autosampler (Palo Alto, California, USA) at 400 and 100 MHz respectively. Deuterated chloroform ($CDCl_3$) and Tetramethylsilane (TMS) were used as solvent and internal standard respectively. 10 mg/0.7 µl (1H) and 25 mg/0.7 µl (^{13}C) of sample in 5 mm NMR tubes were used to carry out the experiment. Coupling constants (J) and chemical shifts (δ) were expressed in Hertz (Hz) and Parts Per Million (ppm), reported relative to residual peaks. Low resolution mass spectra were obtained using TSQ Quantum Access Max (Triple Quadrupole) LCMS/MS in positive ion mode.

Inhibition of nitric oxide production in lipopolysaccharide-activated murine macrophage raw 264.7 cells (iNOS) assay

The inhibitory effects of samples on NO production were evaluated in LPS-activated murine macrophage RAW 264.7 cells by following a previously described method [15]. Briefly, RAW 264.7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with penicillin G sodium (100 units/ml), streptomycin sulfate (100 µg/ml), amphotericin B (0.25 µg/ml), and 10% Fetal Bovine Serum (FBS). The cells were seeded in 96-well culture plates at a density of 1 × 105 cells/well and allowed to adhere for 24 h at 37˚C in humidified CO2 (5%) incubator. Samples dissolved in phenol red-free DMEM were added in the wells, and after 30 min, followed by 1 µg/ml of LPS treatment for 20 h. The concentration of NO in the cultured medium was measured by addition of Griess reagent (90 µl of 1% sulfanilamide and 90 µl of 0.1% N-(1-naphthyl) ethylene diamine in 2.5% H_3PO_4) in each well. The standard curve was created by using known concentrations of sodium nitrite, and the absorbance was measured at 540 nm. L-Na-Monomethyl arginine citrate (L-NMMA) was used as positive control in this assay. Moreover, to evaluate the cytotoxic effect of samples in RAW 264.7 cells under assay conditions, Sulforhodamine B (SRB) assay was also performed. Briefly, cells were fixed with 10% trichloroacetic acid (TCA) after which these were stained with 0.4% SRB solution in 1% acetic acid, followed by addition of 10 mM Tris-buffer for dissolving bound SRB dye. The optical density was determined at 515 nm using a microplate reader (Biotek).

Tumor Necrosis Factor-α Activated Nuclear Factor-κB Inhibition Assay

Stable transfected human embryonic kidney cells 293 (Panomics, Fremont, CA, USA) were utilized in current assay, according to previously described procedure [16]. The cells were maintained in DMEM (Invitrogen Co.; Carlsbad, CA, USA), supplemented with 10% FBS, 100 unit's/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine and afterwards seeded in 96-well plates at a density of 2 × 104 cells/well. TNF-α (human, recombinant, *E. coli*, Calbiochem, Gibbstown, NJ, USA) was used as an activator at a concentration of 2 ng/ml (0.14 nM). After incubation time of 48 h, the medium was replaced, and the cells were treated with test samples (20 µg/ml containing 1% DMSO in PBS). Cells were incubated with tested samples for 6 h, after which the reaction was stopped by adding 50 µl of Reporter Lysis Buffer (Promega, Madison, WI, USA) and frozen overnight at -80˚C. Cells were then thawed, and the inhibitory activity was measured

using a LUMIstar Galaxy luminometer (BMG, Offenburg, Germany) and Luc assay system from Promega (Madison, WI, USA). The gene product, luciferase enzyme, reacts with luciferase substrate, and the emitted light was detected by the luminometer. Data were calculated as percent inhibition. The samples that showed more than 60% inhibition at 20 µg/ml were tested at different concentrations to find IC_{50}. Nα-Tosyl-Lphenylalanine Chloromethyl Ketone (TPCK) was used as a positive control. To avoid false positive results due to cytotoxic effects on samples, a cytotoxicity assay was run simultaneously. The procedure was the same as above, except that instead of 96 white walled well plates, 96 well transparent plates were used, and after 6 h of incubation, the cells were treated with 50 µl of 20% TCA and incubated at 4˚C for 30 min. TCA was then removed and the cells were washed four times with tap water. After keeping the plates overnight for drying, 100 µl of 0.4% SRB in 1% acetic acid was added to each well for 30 min at room temperature. All wells were then washed four times with 1% acetic acid and again air-dried overnight. To each well 200 µl of 10 mM Tris base (pH 10) was added, which was mixed for 10 min on a gyratory shaker to solubilize the bound SRB. The optical density was measured using a micro plate reader (Biotek) at 515 nm, and the percent survival was determined.

Protein Kinase Inhibition Assay

The protein kinase inhibition assay was performed by observing hyphae formation inhibition in purified isolates of *Streptomyces* 85E strain [17]. Microbial lawn was formed by spreading spores and mycelial fragments (100 µl) of 24 h refreshed culture of *Streptomyces* on petri plates containing minimal ISP4 medium under sterile conditions. Sterile 6 mm filter paper discs loaded with 5 µl of each sample (10 mg/ml in DMSO, 50 µg/disc) were placed on the surface of the plates seeded with *Streptomyces* 85E. Surfactin (5 µl of 4 mg/ml in DMSO) and DMSO loaded discs served as positive and negative controls respectively. The plates were then incubated at 30˚C (time required for hyphae formation in *Streptomyces* 85E) for 72-96 h. Results were recorded for the presence of bald or clear zones of inhibition around samples and control discs.

Statistical Analysis

Data are expressed as mean ± SD from triple investigation. Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey test using the Origin Pro 8.5.0 SR1 software (OriginLab Corp., USA). Results were considered significant at $p < 0.05$. IC_{50} values of active samples were calculated by Table Curve 2D Windows version 4.07 (SPSS Inc., Chicago, IL, USA).

Results

Friedelin which is a feidelane-type triterpenoid was isolated as colorless needle like crystals. ESI-MS m/z 426 [M]+; ^{1}H NMR, δ: 0.71 (3H, s), 0.85 (3H, s), 0.87 (3H, d, J = 7.0 Hz, H3-23), 0.93 (3H, s), 0.98 (3H, s), 0.99 (3H, s), 1.03 (3H, s), 1.16 (3H, s), 1.92-2.04 (1H, m, H-4), 2.21-2.30 (2H, m, H2-2). Epifriedelinol was isolated as white sand like crystals. EI-MS m/z 428 [M]+. 1H NMR, δ: 0.85 (3H, s), 0.87 (3H, d, J = 7 Hz, H3-23), 0.93 (3H, s), 0.95 (3H, s), 0.98 (3H, s), 0.99 (3H, s), 1.03 (3H, s), 1.16 (3H, s). The proton of hydroxyl residue gave peak at 2.15 ppm. ^{13}C-NMR data of both triterpenoids have been presented in (Table 1).

Position	Chemical Shifts, δ (ppm)	
	Friedelin	Epifriedelinol
1	22.25	15.75
2	41.5	32.28
3	213.19	72.72
4	58.19	49.12
5	42.12	37.78
6	41.25	41.24
7	18.2	17.51
8	53.07	53.15
9	37.41	37.41
10	59.44	61.29
11	35.98	35.51
12	30.48	30.6
13	39.67	39.66
14	38.26	38.26
15	32.39	32.38
16	35.6	35.97
17	29.96	29.96
18	42.76	42.76
19	35.31	35.31
20	28.14	28.14
21	32.74	32.72
22	39.22	39.22
23	6.8	11.6
24	14.63	16.36
25	17.92	18.21
26	20.23	20.09
27	18.64	18.63
28	32.06	32.28
29	34.5	35.13
30	31.75	31.75

Table 1: ^{13}C-NMR data of triterpenoids isolated from *Q. dilatata.*

The NMR data were compared with previously reported literature [12,18] to determine the structures of isolated compounds, which have been shown in (Figure 1).

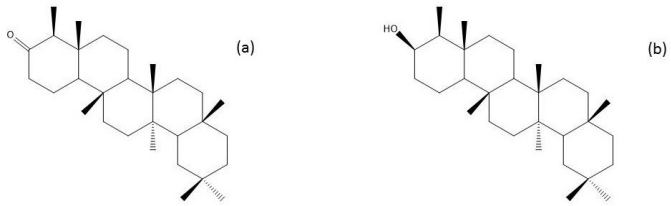

Figure 1: Chemical structures of the triterpenoids; (a) friedelin, (b) epifriedelinol, isolated from *Q. dilatata*.

In chemo-preventive bioassays, friedelin showed enhanced NO inhibition at 20 µg/ml (63.0 ± 5.3%, IC_{50} 13.2 ± 2.7 µg/ml) whereas epifriedelinol exhibited weak inhibitory activity (12.0 ± 6.2%) in nitrite inhibition assay. On the other hand, in SRB cytotoxicity assay, performed to avoid false positive results, both compounds showed > 90% survival rate at 20 µg/ml. The results have been presented in Table 2.

In the present study, friedelin showed good NF-κB inhibitory activity (65.2 ± 3.2%, IC_{50} 18.6 ± 2.2 µg/ml) as compared to epifriedelinol (54.3 ± 4.1%). SRB cytotoxicity assay conducted to avoid false positive results showed > 75% survival rate by both compounds. Results have been presented in (Table 2). These findings are in agreement to previous report in which both compounds attributed to inhibition of IKKβ activated NF-κB in *in vitro* and *in silico* models [19].

Both these triterpenoids were moderately active against the test specie in PK inhibition assay. Friedelin produced 7.0 ± 0.5 mm bald zone, whereas epifriedelinol produced 6.5 ± 0.2 mm bald zone as compared to surfactin (30.0 ± 2.0 mm) (Table 2). Absence of bald or clear zones around negative control impregnated discs confirmed the absence of activity of DMSO.

Samples	NF-κB inhibition assay			Nitrite inhibition assay		Protein kinase inhibition assay	
	% Inhibition	IC_{50} (µg/ml)	% Survival	% Inhibition	% Survival	Clear zone (mm)	Bald zone (mm)
Friedelin	65.2 ± 3.2	18.6 ± 2.2	100.0 ± 8.4*	63.0 ± 5.3	92.3 ± 2.3*	-	7.0 ± 0.5
Epifriedelinol	54.3 ± 4.1	-	92.1 ± 9.2*	12.0 ± 6.2	79.8 ± 1.3*	-	6.5 ± 0.2

Table 2: Results of chemo-preventive assays performed on triterpenoids isolated from *Q. dilatata*.

Values are presented as mean ± standard deviation from triplicate investigation. -: no activity. % inhibition and % survival was calculated at 20 µg/ml final concentration of test samples. Zones of inhibition in protein kinase inhibition assay were presented in mm at 50 µg/disc. The IC_{50} values of positive controls were 10.8 ± 1.7 µg/ml (TPCK), 25.1 ± 2.3 µg/ml (L-NMMA) in case of nitrite and NF-κB inhibition assays respectively, whereas surfactin produced 30.0 ± 2.0 mm, bald zone of inhibition in PKI assay. Statistically significant differences in means ($p < 0.05$) are expressed by * as compared to negative controls in all three assays.

Discussion

Continuous exploration of natural products particularly medicinal plants has undoubtedly led to obtain a number of cytotoxic and chemo-preventive compounds. These compounds may be assumed to have reduced side effects due to established folklore consumption of plant receptacles. It prompts us to persuade the exploitation of these sources for the isolation of novel anticancer and chemo-preventive agents. In the present study, epifriedelin and friedelin obtained from *Q. dilatata* were employed to determine their chemo-preventive potential.

In iNOS assay, LPS-activated murine macrophage RAW 264.7 cells were utilized to assess the inhibition of NO, serving as an indirect marker to monitor iNOS activity. The nitric oxide is a diatomic free radical molecule, generated in mammalian cells using L-arginine, by a family of NO synthases requiring a panel of factors and co-factors to be fully functional [20]. NO is highly reactive, can react with other free radicals, molecular oxygen and heavy metals within biological systems as well as can cause DNA damage by producing nitrites and peroxynitrites. Various factors such as cytokines (e.g. Interferon-γ (IFN- γ), Interleukin-1 (IL-1) and Tumour Necrosis Factor-α (TNF-α), bacterial endotoxin (LPS) and oxidative stress (e.g. under conditions encountered during hypoxia) are responsible for the regulation of iNOS expression [20]. iNOS is not only expressed in stromal cells along with peritumoral and intratumoral macrophages, but also in tumor cells themselves in invasive breast carcinomas [21]. The iNOS gene expression has also been reported in various other carcinomas such as brain, head and neck, esophagus, lung, prostate, bladder, pancreatic cancers [20]. Further, the observations of iNOS overexpression during early tumor developmental stages in these organs demonstrate possible usage of iNOS inhibitors in cancer chemoprevention [22].

NF-κB is a family of transcription factors including 5 genes i.e. NF-κB1 (p50/p105), NF-κB2 (p52/p100), RelA (p65), c-Rel and RelB. NF-κB is activated by various signaling pathways, triggered

by several activation factors such as tyrosine kinases, cytokinens as well as enhanced expression of members of epidermal growth factors and Tumor Necrosis Factors (TNF-α) families [23]. The activation of NF-κB gene family is strictly a tightly regulated event; however different molecular alterations can result in impaired regulation and activation of genes under NF-κB control. These genes are responsible for various cellular processes including division, apoptosis, adhesion, migration etc., the deregulation of which can lead to carcinogenesis [23]. Several animal models have proved the linkage between NF-κB activation and cancer initiation [24]. Further its expression has also been observed in breast, nasopharyngeal, colon, ovarian, pancreatic, bladder, prostate carcinomas and melanoma [23]. Numerous NF-κB inhibitors have been reported so far including some natural compounds from medicinal plants such as cudratricusxanthone from *Cudriana tricuspidata*, curcumin from *Curcuma longa*, trans-resveratrol from grapes, or withanolides from *Withania coagulans* [25,26]. Research for potent inhibitors of this arch nemesis is still in perpetuation, provoking us to scrutinize isolated compounds from said plant.

In eukaryotic genome, Protein Kinase (PK) family is considered as one of the largest gene families which are responsible for the phosphorylation of either serine-threonine (ser/ther specific protein kinase, STPKs), tyrosine (tyrosine specific protein kinases, TPKs) and both threonine and tyrosine (dual specific protein kinase) [27]. Alterations in regulatory signaling pathways due to mutations of these genetic components can lead to tumor initiation, invasion, angiogenesis and progression [28]. Thus, Protein Kinase Inhibitors (PKIs) can serve as potential anticancer or chemo-preventive agents. Due to enhanced toxicity levels and reduced therapeutic profiles of existing chemotherapeutic measures, quest for the identification of specific inhibitors of these deregulated PKs is in continuance. Over 20 different PKIs are currently being used in modern anticancer regimens, but enhanced demand encourages us to discover novel PKIs [29]. Current study involves *Streptomyces* 85E strain as a model to determine protein kinase inhibitory activities of friedelin and epifriedelinol; as signal transduction mechanisms of this particular specie involve eukaryotic-type protein phosphorylation for the growth of multicellular filamentous hyphae. The inhibition of hyphae formation may be attributed to the inhibition of protein kinases.

Significant bioactivities of friedelin in all the bioassays performed in the current study might be attributed to presence of unsaturation or ketonic bond. This hypothesis is supported previously when the ketone linked benzofuran derivatives demonstrated potent antibacterial activities against both Gram positive and negative bacterial test strains [30]. The substantial bioactivity showed by friedelin; isolated from *Q. dilatata*, makes it an important candidate for further exploitation in mechanistic based analyses as well as synthetic chemistry to prepare compatible analogues of the said compound.

Conclusion

Cancer being the deadliest disease of all times requires novel therapeutic agents for its reversal. Current study was designed to explore the chemo-preventive potential of triterpenoids isolated from *Q. dilatata*. Among the isolated compounds, friedelin was found to be more active as compared to epifriedelinol. These results suggest the need for extensive pharmacological investigations of these metabolites isolated from subject plant. Further lipophilic nature of this compound demands its utilization in synthetic analogues preparation for proper bioavailability.

Competing Interest

The authors declare that they have no competing interest.

Acknowledgement

The authors are thankful to Prof. Dr. Rizwana Aleem Qureshi, Department of Plant sciences, Faculty of Biological sciences, Quaid-i-Azam University Islamabad, Pakistan for identifying the plant sample. We acknowledge the financial support given by Higher Education Commission Pakistan under PhD indigenous fellowship program to finance this research project.

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