Bioassay-guided Isolation of New Antitumor Agent from Ficus faveolata (Wall. ex Miq.)

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Abstract

Ficus species have been used in both Ayurvedic and Traditional Chinese Medicine (TCM); however the medicinal uses of these species are widely found and originated in Middle East. The phytochemical study of Ficus foveolata was undertaken with small scale extraction of stem (300 g) for cytotoxic screening and dereplication purpose. The crude methanolic extract of Ficus was partitioned into different fractions of hexane, dichloromethane and methanol. All the five fractions FA, FB, FC, FD and FE were screened for their anti-proliferative effect in the disk diffusion assay (In vitro) against six cancer cell lines. The bioassay-guided isolation of a new antitumor agent (Ficusonolide; 3α-hydroxyolean-12-en-29, 19α-olide (1)) was carried out from the methanolic extract (FB) of Ficus faveolata. Its structure was elucidated on the basis of extensive spectroscopic techniques (IR, MS and NMR). The pure compound 1 was evaluated against twelve cell cancer lines for the determination of its anti-proliferative potency. For example, in disk diffusion assay the dichloromethane fraction (FB) showed excellent activity at very low concentration. The compound 1 exhibited strong and selective activity against two cancer cell lines: H116 (Human colon adenocarcinoma) and H125 (Human lung adenocarcinoma) with the IC50=7.8 μg/ml and 11.0 μg/ml respectively. The selectivity and potency of the pure compound 1 was in concordance with the activity profile of the fraction FB and ethno-medicinal uses of this plant. This small project on local medicinal plants has opened new vista for future research work on indigenous medicinal plants. The compound 1 can be used a template compound for further studies, as a chemotherapeutic agents against cancer.

Keywords: Moraceae; Ficus foveolata; Stem; Cytotoxicity; Crude fractions; Ficusonolide

Introduction

Ficus (Fig genus) is one of the largest and most important genus of the family Moraceae (mulberry). It consists of more than 800 species with habitats in lowland rainforest of tropical region [1]. Ficus species have been used in both Ayurvedic and Traditional Chinese Medicine (TCM); however the medicinal uses of these species are widely found and originated in Middle East [2]. The different parts (roots, stem, leave, fruits and latex) of Ficus spp. have shown anti-diabetic, anticancer and anti-inflammatory activities [3]. The phytochemical investigation of these species resulted into the isolation of different classes of bioactive secondary metabolites such as phenanthroindolizidine alkaloids, coumarins, multiple flavonoids, triterpenoids, different triacylglycerols and a number of volatile compounds [3-6].

The Ficus foveolata is a scandent climber shrub distributed in most of the Asia [7]. In Pakistan this plant is widely distributed in northern regions (Khyber Pukhtoonkhwa) of Pakistan, where it is locally called ‘baat anzar’ and has wide uses in folk medicines. According to our survey the powdered stem of Ficus foveolata is mixed with other local medicinal plants for the treatment of a cancer type locally called ‘nasoor’ which convinced us for future phytochemical investigation of this plant. Furthermore the Ficus foveolata has also been reported to be used in traditional medicines in the rest of the world for different ailments and disorders. For example, in Nepal its bark is used as lactating agent for milk secretion [8] and in Thailand, people use it as a tonic in a number of ways [9]. Literature survey showed only two recently published articles on the phytochemical constituents of this plant which reported the isolation of (1,2E,2E)-1,2-docosanediol diferulate [10] and two new eudesmane-type sesquiterpenes [11]. In the present study, we explored the selective anti-tumor potential of crude methanolic fractions and pure compound 3α-hydroxyolean-12-en-29,19α-olide (1), along with isolation and characterization studies of compound 1.

The phytochemical study of Ficus faveolata was undertaken with small scale extraction of stem (300 g) for cytotoxic screening and dereplication purpose. The crude methanolic extract was partitioned into different fractions of hexane, dichloromethane and methanol. All the five fractions FA, FB, FC, FD and FE were screened for their anti-proliferative effect by using the disk diffusion assay (In vitro). Initial stimulus for further research on dichloromethane fraction (FB) was its impressive activity against twelve different cancer cell lines (assayed at U.S. Josephine Ford Cancer Center). The LC-MS profile of the FB fraction was developed for dereplication purpose which exhibited few peaks with one major at m/z 454. The known data was dereplicated by comparing the UV and MS data with reported compounds in Dictionary of Natural Product (DNP). A number of hits were observed in DNP for the major peak at m/z 454 however due to interesting 1H NMR
spectrum the peak was selected for purification and characterization. Large scale extraction of *Ficus foveolata* stem (13 kg) resulted into the isolation of 3α-hydroxyolean-12-en-29,19α-olide; Ficusonolide (1, C30H46O3, m/z 454) (Figure 1).

Materials and Methods

General

Melting point was determined using Buchi 535 digital device. Optical rotation was taken on Jasco-P2000 digital polarimeter in MeOH at room temperature while the IR (KBr) data was recorded on Bruker VECTO 22 spectrophotometer; νmax in cm⁻¹. UV data was taken from 996 photodiode array detector connected to analytical HPLC-MS instrument. 1H NMR (300 MHz, C5D5N) and 13C NMR (125 MHz, CD3OD) spectra were recorded on Bruker instrument, the chemical shift value was presented in 6 (ppm) and coupling constant (J) in Hz. For the purity of isolates and chemical profile of fraction FB analytical HPLC (MeOH/H2O with 0.1% FA) equipped with Phenomenex Luna column C18 RP 5µm (5 µm, 150 × 4.6), Sedex 55 ELS detector (ELSD), 996 photodiode array detector and ESI-TOF-MS (+) was used. Phenomenex 5µm Luna C18 RP column (250 × 10) was used for preparative HPLC. ESI-MS and HRESIMS were recorded on mariner ESI-TOF-MS instrument.

Plant material

The stem of *Ficus foveolata* was collected from district Buner, Khyber Pakhtoonkhwa, Pakistan during the month of July 2007. The plant was identified by taxonomist Mr. Ambara Khan and voucher specimen (Bot.15077) was deposited in the Herbarium of Department of Botany, University of Peshawar, Pakistan.

Extraction and isolation

The air dried stem (13 kg) of *Ficus foveolata* were repeatedly extracted (X3) with 80% MeOH/H2O at room temperature after every 24 hrs. The combined extract was concentrated under vacuum at 40°C, resulted into brownish thick syrup that constituted the crude aqueous extract (35 g). The polarity of suspension was changed to 10% MeOH/H2O FA, FB, FC, FD and FE on polarity basis. The suspended crude extract was extracted (X3) with 80% MeOH/H2O at room temperature after every 24 hrs. The combined extract was concentrated under vacuum at 40°C, resulting into brownish thick syrup that constituted the crude aqueous extract (10 g), while the remaining DCM insoluble phase was get concentrated to obtain DCM insoluble phase (7 g). Finally the polarity of suspension was changed to approximately 70% MeOH/H2O FA, FB, FC, FD and FE pure compound 1 (isolated from FB fraction) was screened against six and twelve cancer cell lines respectively. Selectivity is the measure of differential inhibition of solid tumor cell line against normal or leukemia cell line (200 zone units=6.5 mm) in the disk diffusion assay, while positive activity is define as zone of inhibition greater 250 zone units. After dissolution in DMSO, the samples were pipetted onto a paper disk and then applied to an agar plate which is seeded with a particular cell line. Then the agar plates were incubated for cell growth, and the samples activity was analyzed by the size of zone of inhibition (in zone units or mm) of cell growth on agar plate. For determination of IC50 value, human tumor cells were seeded in concentration of 5×104 cells in T25 tissue culture flasks (Falcon Plastics, NJ, USA) with 5 mL media RPMI 1640 (Cellgro, Virginia) supplemented with 15% BCS (Hyclone, Utah), 5% Penicillin/Streptomycin and 5% Glutamine. After three days incubation (cells in logarithmic growth phase; 5×105 cells/flask), test compound was added to the flasks to achieve concentrations ranging from 10−4 to 10−5 μg/mL. The cultured flasks were then incubated for seventy-two hours, then the cells were washed, trypsinized, spun down and counted for both viable and dead cells using 0.08% trypan blue (Gibco, Maryland). The number of viable cells number was plotted as a function of concentration and the IC50 value determined by interpolation [13]. Each point was carried out in duplicate and a standard deviation determined.

Results and Discussion

Compound 1 (Ficusonolide) was isolated as white amorphous powder and its structure elucidation was commenced by developing molecular formula C30H46O3 via high resolution ESIMS which showed molecular ion peak at m/z 454.3456 [M⁺]+ (calcd. for C30H46O3, m/z=455.3447). Molecular formula C30H46O3 of 1 exhibited

![Figure 1: The structure of compound 1 isolated from the stem of *Ficus foveolata*](image-url)
The 1H and 13C NMR spectra displayed signals which were assigned to eight methyl’s [δ C 16.6/δH 1.04 (3H, s, Me-23), δ C 28.7/δH 1.22 (3H, s, Me-24), δ 15.8/δH 0.94 (s, Me-25), δ 21.2/δH 1.21 (3H, s, Me-26), δ 24.0/δH 1.07 (s, Me-27), δ 24.8/δH 0.80 (s, Me-28) and δ 17.1/δH 0.90 (s, Me-30)]. The two oxymethine signals at δ H 3.43 (1H, dd, J = 10.5, 4.0 Hz) and δ H 4.13 (1H, d, J = 5.4 Hz) coupled with δ C 77.9 and δ C 83.1 in the HSQC respectively, and a vinylic methine at δ H 5.30 (1H, t, J = 3.6 Hz) show strong correlation with carbon at δ C 124.9 in the HSQC spectrum and carbon atoms at δ 47.8, δ 39.5 and δ 43.3 in HMBC spectrum.

The 13C NMR indicated the presence of ester group δC 182.3 (C-29) and a trisubstituted double bond δ C 124.9 (C-12) and δ C 140.5 (C-13). These characteristic spectral data suggested the presence of oleane-12-en skeleton in compound 1 [13]. Furthermore the mass spectrum showed standard retro-Diel Alder fragmentation (m/z 207 and 246) (Figure 2c), indicating the presence of lactone ring at ring D and/or E [14]. The presence of one carbonyl group, one olefinic bond and six rings in compound 1 can fully satisfied eight degree of unsaturations evident in the molecular formula C_{30}H_{46}O_{3}. All these spectral data suggested that compound 1 was olean-12-en type triterpene with a secondary hydroxyl group and lactone ring. However the position and spatial orientation of hydroxyl group and lactone ring were yet to have determined.

The attachment of hydroxyl group at position C-3 and lactone ring on ring E (between C-17 and C-19) were made by 2D correlation spectra (COSY and HMBC) and mass fragmentation pattern (Figures 2a-2c). The connection of downfield resonating proton at δ H 3.43 (t, J =3.0 Hz; H-3) with carbon at δ C 77.9 (C-3) was observed in the HSQC correlation spectrum, indicating the attachment of a hydroxyl group at C-3. This assignment was confirmed by HMBC correlations of Me-23 (δ C 140.5) and Me-24 (δ C 83.1) with C-3 (δ C 79.7), C-4 (δ C 38.0) and C-5 (δ C 48.0), while long range HMBC correlations were observed for protons of C-2 (Ha, m, 2.01, Hb, m, 1.18) with C-3 (δ C 79.7), C-5 (δ C 48.0) and C-25 (δ C 16.1). The attachment of hydroxyl group at C-3 was further supported by COSY correlations of proton at δ H 2.01 (m, H-2) with δ C 3.43 (dd, J =10.5, 4.0 Hz; H-3) and δ 2.23 (m, H-1).

Mass fragmentation of compound 1 indicated the presence of lactone ring in either ring D and/or E, however the exact attachment of lactone ring to ring E (between C-17 and C-19) was made by COSY and HMBC correlations (Figures 2a-2c). HMBC spectrum exhibited long range correlations of proton at δ H 4.13 (d, J =5.4 Hz; H-19) with carbons at δ C 42.6, 24.8, 182.3 and 17.1, while proton at δ H 1.95 (m, H-21) with carbons resonating at δ C 42.6, 83.1 and 17.1. COSY spectrum showed correlations of proton at δ H 4.13 (d, J =5.4 Hz; H-19) with δ C 140.5 (s; H-18) and δ C 83.1 (s; H-28) (Figures 2a-2c).

The relative configuration of hydroxyl group at C-3 was determined...
by splitting pattern, coupling constant and NOESY correlations. The carbinol proton in compound 1 appearing as doublet of doublet with large coupling constant at δ 3.43 (dd, J=10.5, 4.0 Hz; H-3) which revealed the equatorial positions of hydroxyl group at C-3 instead of triplet for axial position. The NOESY spectrum showed axial-equatorial correlations of proton δ 3.43 (H-3) with δ 1.18 (H-2) and axial-axial correlation with δ 2.23 (β-H-1), similarly proton δ 2.01 (β-H-2) displayed correlations with δ 1.82 (α-H-1) and δ 1.04 (Me-23) (Figure 2d) further verified that hydroxyl group at C-3 was equatorially oriented.

The relative stereochemistry of rings E and lactone were developed on the basis of NOESY spectrum and protons splitting pattern. Proton resonated at δ 4.13 (J=5.4 Hz; H-19) appeared as doublet indicating its axial position instead of equatorial which appeared as doublet of doublet in 3-epiabrusalactone [14]. The NOESY spectrum showed correlations of proton at δ 4.13 (H-19) with δ 0.90 (αMe-30) and δ 1.63 (β-H-22), and proton at δ 3.50 (H-18) with δ 0.85 (αH-22) and δ 0.80 (βMe-28) (Figure 2d). On the basis of spectral data and comparison with spectral data of related compounds [15], the structure of 1 was elucidated as 3α-hydroxyolean-12-en-29,19α-olide (Ficusonolide), which to the best of our knowledge is a new compound.

In the preliminary screening, all the five crude fractions (FA-FE) were screened for their cytotoxicity against six cancer cell lines on different dilution (Table 2). All the fractions exhibited variable degree of cytotoxic activity. Initial results indicated that fractions (FA and FC) did not showed reasonable cytotoxic activity compared to FB, FD and FE fractions. For further confirmation, the fractions FB, FD and FE were also evaluated on high dilution. The fraction FD was moderately active with zone of inhibition of 750, 350, 550, 700 and 750 (200 zone units=6.5 mm) for L1210, Colon38, CFU-GM, H-116 and H-125 cell lines respectively. The fraction FD were also checked on 1/4 dilution, where at low concentration the activity declined approximately at 300 zone of inhibition. The fraction FE was found to show significant activity with zone of inhibition 900 against Murine lymphocytic leukemia. At low concentrations (dilution of 1/4 and 1/16), the FE exhibited reasonable activity with zone of inhibition 800 and the activity was declined to the zone of inhibition approximately 550 at dilution of 1/64 for all cell lines. The fraction FB was found to be most significantly active among all five fractions having zone of inhibition more than 1000 for all cell lines. Interestingly the fraction FB was found consistently active even up to low dilution (1/64 dilution) exhibiting zone of inhibition more than 1000. The FB showed significant inhibition at dilution 1/256 with zone of inhibition more than 1000 against both L1210 and H-125. The activity of this fraction (FB) retain consistent at dilution of 1/4096 against H116 and H-125 with zone of inhibition 700 and 1000 respectively. The fraction FB also showed significant inhibition at very dilution 1/16384 against H-125 (Table 2). For the reason of significant inhibition of fraction FB against these cell lines especially against H116 and H-125, the fraction FB were subjected to column chromatography, as a result the compound ficusonolide (1) was identified.

Ficusonolide (1) was tested against twelve cancer cell lines (Table 3). Interestingly, Ficusonolide (1) was also found active against two H116 cells (Human colon adenocarcinoma) and H125 cells (Human lung adenocarcinoma) with IC50 values 7.8 and 11.0 μg/mL respectively. The activity results of compound 1 were in harmony with the activity profile of the fraction FB and ethno medicinal uses of this plant. This small project on local medicinal plants has opened new vista for future research work on indigenous medicinal plants.

**Cancer cell line** | **IC50 in μg/mL**
---|---
L1210 (Murine lymphocytic leukemia) | >100
Colon38 (Murine colon adenocarcinoma) | >100
CFU-GM (Murine granulocyte macrophage colony formy unit) | >100
H116 (Human colon adenocarcinoma) | 7.8
H125 (Human lung adenocarcinoma) | 11.0
MCF-7 (Hormone responsive breast cancer) | >100
LNCaP (Androgen sensitive prostate cancer) | >100
OVC-5 (Ovarian cancer) | >100
U251N (Glioblastoma) | >100
MDA (Melanoma) | >100
PANC-1 (Marine pancreatic solid tumor) | >100
CEM (Humanleukemic lymphoid) | >100

**Table 3:** Anti-proliferative activity (IC50 in μg/mL) of Ficusonolide (1).

The bioassay guided chemical investigation of the Ficus faveolata stem resulted in the isolation of a new compound 1; 3α-hydroxyolean-12-en-29,19α-olide (Ficusonolide). All the crude methanolic fractions (FA, FB, FC, FD and FE) and pure compound 1 were screened for their cytotoxic activity against six and twelve cancer cell lines respectively. The selectivity and potency of the pure compound 1 were in harmony with the activity profile of the fraction FB and ethno medicinal uses of this plant. This small project on local medicinal plants has opened new vista for future research work on indigenous medicinal plants.

**Conclusions**

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