Journal of Cancer Science & Therapy

Din et al., J Cancer Sci Ther 2013, 5.11
DOI: 10.4172/1948-5956.1000233

Research Article Open Access

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 under taken 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-hydroxylean-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. 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 IC_{50}=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 ailment 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*E*,22*E*)-1,22-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 3*α*-hydroxylean-12-en-29,19*α*-olide (**1**), along with isolation and characterization studies of compound **1**.

The phytochemical study of *Ficus foveolata* was under taken 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 ^{1}H NMR

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Received October 03, 2013; **Accepted** November 18, 2013; **Published** November 23, 2013

Citation: Din AU, Uddin G, Hussain N, Khan A, Khan I, et al. (2013) Bioassay-guided Isolation of New Antitumor Agent from Ficus faveolata (Wall. ex Miq.). J Cancer Sci Ther 5: 404-408. doi:10.4172/1948-5956.1000233

spectrum the peak was selected for purification and characterization. Large scale extraction of *Ficus foveolata* stem (13 kg) resulted into the isolation of 3α-hydroxylean-12-en-29,19α-olide; Ficusonolide (**1**, $C_{30}H_{46}O_3$, *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 VECTOR 22 spectrophotometer; ν_{max} in cm^{-1}. UV data was taken from 996 photodiode array detector connected to analytical HPLC-MS instrument. ^1H NMR (300 MHz, C_5D_5N) and ^{13}C NMR (125 MHz, C_5D_5N) spectra were recorded on Bruker instrument, the chemical shift value was presented in δ (ppm) and coupling constant (*J*) in Hz. For the purity of isolates and chemical profile of fraction FB analytical HPLC (MeOH/H_2O with 0.1% FA) equipped with Phenomenex Luna column C18 RP (5µm, 150 × 4.6), Sedex 55 ELS detector (ELSD), 996 photodiode array detector and ESI-TOF-MS (+) was used. Phenomenex 5µm Luna C_{18} 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 faveolata* was collected from district Buner, Khyber Pukhtoonkhwa, 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 faveolata* were repeatedly extracted (X3) with 80% MeOH/H_2O at room temperature after every 24 hrs. The combined extract was concentrated under vacuum at 40°C, to obtain brownish thick syrup that constituted the crude aqueous methanolic extract (100 g) which was first partitioned into five fractions: FA, FB, FC, FD and FE on polarity basis. The suspended crude extract in water was defatted (X3) with petroleum ether affording fraction FA (35 g). The polarity of suspension was change to 10% MeOH/H_2O followed by extraction with dichloromethane (DCM) to obtain DCM soluble fraction FB (11.5 g). By addition of MeOH (1.8 L) the polarity of aqueous suspension was again changed to 50% MeOH/H_2O and extracted with DCM to obtain fraction FC (7 g). Finally the polarity of the aqueous layer was changed to approximately 70% MeOH/H_2O which was further extracted with DCM to get DCM soluble fraction FD (10 g), while the remaining DCM insoluble phase was get concentrated under vacuum to get methanolic fraction (FE).

After all the five fractions were screened for cytotoxic activity against six cancer cell lines, the LCMS profiles of fraction FB was developed. Fraction FB (11.5 g) was subjected to HPLC (Phenomenex Luna column C_{18} RP (5 µm, 150 × 4.6; 0.1 % acidic (formic acid) gradient solvent system (10-100 MeCN/H_2O in 30 minutes) in order to purified the selected peak, which resulted into the isolation of compounds **1** (14 mg).

Physical data of compound 1

White amorphous powder with molecular formula $C_{30}H_{46}O_3$ (14 mg); mp 177 °C; $[\alpha]_D^{29.7}$117 (c 0.1, MeOH); IRfilm (KBr): 3490, 3050, 1748 cm^{-1}; EIMS *m/z* (rel. int.%): 246.1 (100), 190 (50), 207 (40), 454 $[M]^+$ (17); HRESIMS *m/z* 455.3456 $[M^+ + H]$ (calcd. 455.3447 for $C_{30}H_{46}O_3$); for ^1H and ^{13}C NMR Table 1.

Anti-proliferative *in vitro* disk diffusion assay

The Disk Diffusion Assay (DDA) was performed in Josephine Ford Cancer Center (JFCC) [12]. For determination of cytotoxic potency and solid tumor selectivity, the crude fractions (FA, FB, FC, FD and FE) and pure compound **1** (isolated from FB fraction) were 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 IC_{50} value, human tumor cells were seeded in concentration of 5×10^4 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; 5×10^5 cells/flask), test compound was added to the flasks to achieve concentrations ranging from 10^1 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 IC_{50} 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 $C_{30}H_{46}O_3$ *via* high resolution ESIMS which showed molecular ion peak at *m/z* 455.3456 $[M+H^+]$ (calcd. for $C_{30}H_{46}O_3+H^+$=455.3447). Molecular formula $C_{30}H_{46}O_3$ of **1** exhibited

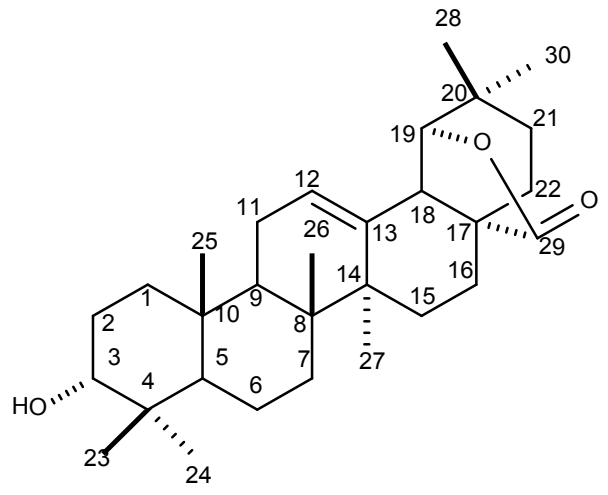

Figure 1: The structure of compound **1** isolated from the stem of *Ficus foveolata*.

C. No	δC	δH/ppm (multi, integral, J/Hz)	HMBC
1	33.8	2.23 (1H, *s*)	C3,C5
		1.82 (1H, *m*)	C4, C10
2	25.6	2.01 (1H, *m*)	
		1.18 (1H, *m*)	
3	77.9	3.43 (IH, *dd*, 10.5 and 4.0)	
4	37.3		
5	55.7	2.03(1H, *m*)	C8, C10
6	18.7	1.65(1H, *d*, 3.6)	
		1.45 (1H, *d*, 3.0)	C5, C9, C14
7	33.4	1.46 (1H, *m*)	
		1.34 (1H, *m*)	
8	39.6		
9	47.8	1.57 (1H, *m*)	
10	35.4		
11	23.7	1.84 (1H, *m*)	C8, C13
		1.20 (1H, *m*)	
12	124.9		
13	140.5		
14	39.5		
15	28.1	1.80 (1H, *m*)	
16	24.6	0.99 (1H, *m*)	C14, C18
		1.57 (1H, *m*)	
17	42.6		
18	43.3	0.85 (1H, *s*)	C14, C16
19	83.1	4.13 (1H, *d*, 5.4)	
20	39.3		
21	40.3	1.95 (1H, *m*)	
		1.47 (1H, *m*)	
22	39.1	1.63 (1H, *m*)	
		1.52 (1H, *m*)	
23	16.6	1.04 (3H, *s*)	C3, C5
24	28.7	1.22 (3H, *s*)	C3, C5
25	15.8	0.94 (3H, *s*)	C1,C 9
26	21.2	1.21 (3H, *s*)	C9, C14
27	24.0	1.07 (3H, *s*)	C7, C15
28	24.8	0.80 (3H, *s*)	C16, C22
29	182.3		
30	17.1	0.90 (3H, *s*)	C19, C21, C29

Table 1: ^{1}H and ^{13}C NMR of ficusonolide (**1**) (500 MHz (^{1}H NMR) and 125 MHz (^{13}C), C_5D_5N, *δ* in ppm, *J* in Hz).

8 degree of unsaturations and its IR spectrum displayed strong absorption bands at 3490, 2945, 1748 and 1377 cm^{-1}, indicating the presence of hydroxyl, C-H saturated ester carbonyl and *gem*-dimethyl functionalities respectively. The ^{13}C NMR spectrum of **1** indicated 30 carbon atoms including seven methyls, nine methylenes, six mithines and eight quaternary on the basis of DEPT spectrum analysis (Table 1). The ^{1}H and ^{13}C NMR spectra displayed signals which were assigned to eight methyl's [δ_C 16.6/δ_H 1.04 (s, Me-23), δ_C 28.7/δ_H 1.22 (s, Me-24), δ_C 15.8/δ_H 0.94 (s, Me-25), δ_C 21.2/δ_H 1.21 (s, Me-26), δ_C 24.0/δ_H 1.07 (s, Me-27), δ_C 24.8/δ_H 0.80 (s, Me-28) and δ_C 17.1/δ_H 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_C 47.8, δ_C 39.5 and δ_C 43.3 in HMBC spectrum.

The ^{13}C NMR indicated the presence of ester group δ_C 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_H 1.02) and Me-24 (δ_H 1.22) 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 2.01 (m, H-2) with δ 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_H 0.85 (brd. s; H-18) and δ_H 0.80 (s, Me-28) (Figures 2a-2c).

The relative configuration of hydroxyl group at C-3 was determined

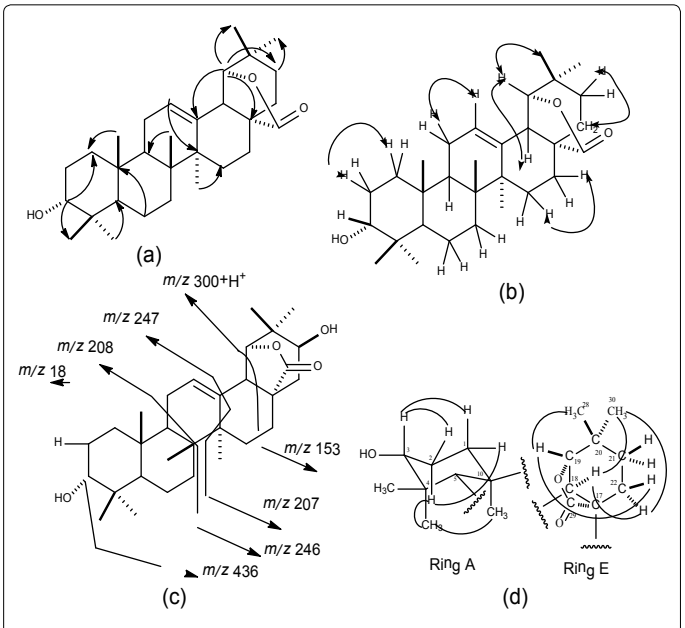

Figure 2: (a) Important HMBC correlations, (b) COSY correlations, (c) mass fragmentation pattern and (d) NOESY correlations.

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 (*α*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_H 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-*epi*abruslactone [14]. The NOESY spectrum showed correlations of proton at δ_H 4.13 (H-19) with δ_H 0.90 (*α*Me-30) and δ_H 1.63 (*β*H-22), and proton at δ_H 0.85 (H-18) with δ_H 0.85 (*α*H-22) and δ_H 0.80 (*β*Me-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 3*α*-hydroxylean-12-en-29,19*α*-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 moderatively 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 line 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 lyphocytic 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

Fract[a]	Dil[b]	L1210	Colon38	CFU-GM	H-116	H-125	CEM
DMSO	0	0	0	0	0	0	0
FA		550	300	300	400	600	500
FB		>1000					
"	1/4	>1000					
"	1/16		>1000				
"	1/64				>1000		
"	1/256	>1000	500			1000	
"	1/1024			700	900	>1000	650
"	1/4096			800	700	>1000	500
"	1/16384			450		850	350
FC		350	350	300	200	300	450
FD		750	350	550	700	750	
	1/4	450	100				
FE		900					
	1/4	800					
	1/16	800	350	450			
	1/64			550	400	450	550

[a]Fract=Crude fractions, [b]Dil=Dilution, L1210 (Murine lyphocytic leukemia), Colon38 (Murine colon adrenocarcinoma), CFU-GM (Murine granulocyte macrophage colony formy unit), H-116 (Human colon adrenocarcinoma), H-125 (Human lung adrenocarcinoma) and CEM (Humane leukemic lymphoid)

Table 2: *In vitro* disk diffusion assay for crude fractions (FA-FE).

Cancer cell line	IC_{50} in µg/mL
L1210 (Murine lyphocytic leukemia)	>100
Colon38 (Murine colon adrenocarcinoma)	>100
CFU-GM (Murine granulocyte macrophage colony formy unit)	>100
H116 (Human colon adrenocarcinoma)	7.8
H125 (Human lung adrenocarcinoma)	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 (Murine pancreatic solid tumor)	>100
CEM (Humane leukemic lymphoid)	>100

Table 3: Anti-proliferative activity (IC_{50} in µg/mL) of Ficusonolide (**1**).

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 upto 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 adrenocarcinoma) and H125 cells (Human lung adrenocarcinoma) with IC_{50} values 7.8 and 11.0 µg/mL respectively. The activity results of compound **1** were quite consistent with the trend observed for crude fraction FB from which leaded to the isolation of compound **1**. The literature survey for the comparison and structure activity relationship did not show same activity for other lactones with similar skeleton.

Conclusions

The bioassay guided chemical investigation of the *Ficus faveolata* stem resulted in the isolation of a new compound **1**; 3*α*-hydroxylean-12-en-29,19*α*-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.

Acknowledgement

This work was financially supported by Higher Education Commission (HEC) of Pakistan. We would like to thank Mr. Ambara khan for taxonomic identification plant and university of California Santa Cruz, USA for their help in providing research facilities.

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Citation: Din AU, Uddin G, Hussain N, Khan A, Khan I, et al. (2013) Bioassay-guided Isolation of New Antitumor Agent from Ficus faveolata (Wall. ex Miq.). J Cancer Sci Ther 5: 404-408. doi:10.4172/1948-5956.1000233

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