

Original Research Article

Triterpenoids with Cytotoxic Potential from the Leaves of *Tridax procumbens* L.

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Abstract

The anticancer activity of crude extracts of the leaves of *Tridax procumbens*, against two breast cancer cell lines-MCF-7 (benign) and MDA-MB-231 (metastatic) were investigated and an attempt was made to identify the anticancer principle. The extracts with methanol (TPM), ethanol (TPE) and chloroform (TPC) as solvents were screened for cytotoxicity by MTT assay against MCF-7 cells. The effective extract was further evaluated on MDA-MB-231 cells. Among the three extracts, TPC was effective at an IC₅₀ value of 136 µg/ml and 129 µg/ml on MCF-7 and MDA-MB-231 cells respectively. Phytochemical analysis of the extract showed the presence of only steroids and terpenoids and their concentration was high (77.4% w/w). HPLC-MS of the chloroform soluble crude extract revealed a major peak (57.59% concentration) at a retention time of 4.78 min and MS data of this peak revealed presence of two fragments of molecular weight 475.80 and 701.80. The compounds were identified to be 3β, 9β-Dihydroxy-18-oleanan-28-oic acid, a derivative of oleanolic acid and 3β, 28-Bis (cinnamoyl) betulin respectively, both from the triterpenoid family.

Keywords: *Tridax procumbens*, cytotoxicity, MCF-7, MDA-MB-231, Triterpenoids.

Introduction

Plants are an important source of anticancer drugs. Almost 50% of the anticancer drugs are either natural plant products or synthetic analogs [1]. For example, paclitaxel for ovary, breast and lung cancers; vincristine for leukemia; podophyllotoxin for lymphomas and camptothecin for ovarian cancer [2].

Tridax procumbens Linn. (*T. procumbens* L.) from the family Asteraceae is commonly known as 'Ghamra' in local language and 'coat buttons' in English. The plant is endemic to tropical regions of Asia, America, Africa and Australia. In India, *Tridax* is a widely distributed weed. It is seen along the roadsides, waste grounds, dikes, railroads, riverbanks, meadows, and dunes [3]. This herb/weed is known to have a wide range of pharmacological activities which have been used in the traditional Indian systems of medicine like Ayurveda, Sidhha and Unani [4]. In Ayurvedic medicine in particular, *Tridax* is a component of "Bhringraj", which is a composition used in treatment of various disorders [5]. The wide range of therapeutic applications is attributed to the presence of phytochemicals such as alkaloids, carotenoids, flavonoids, terpenoids, saponins and tannins [4]. Compounds with anticancer property such as β-sitosterol, β-sitosterol-3-O-β-D-xylopyranoside glucoluteolin, quercetin, luteolin, and procumbenetin have been isolated / purified from the leaves of *T. procumbens* [6]. Vishnupriya

et al. 2011 have demonstrated the cytotoxicity of the aqueous and acetone extracts of leaf and flower of this plant in PC 3 human prostate epithelial cancer cell line [7, 8].

The present work aims to screen the methanol, ethanol and chloroform soluble crude extracts of the leaves of *T. procumbens* L., for anticancer activity against two breast cancer cell lines-MCF-7 (benign) and MDA-MB-231 (metastatic) and to identify the anticancer principle.

Materials and Methods

Plant material

Leaves of *T. procumbens* were collected in Mangalore (Karnataka, India) and identified by a taxonomist, Department of Botany, St. Agnes College, Mangalore, India. A voucher specimen (SAC/BOT-15/108) is deposited at the Herbarium of Department of Botany, St. Agnes College, Mangalore, India.

Preparation of extracts

Fresh leaves were collected, washed with distilled water, shade dried and cut into small pieces. These dried samples were powdered and stored at 4 C until further use. Three crude extracts

(10% w/v) were made using solvents, i.e., methanol, ethanol and chloroform, by maceration for 2 hours with intermittent shaking. The extracts were filtered through what man no. 1 and the clear filtrate was evaporated to dryness to form methanol soluble (TPM), ethanol soluble (TPE) and chloroform soluble (TPC) crude extracts respectively. The yield of the extracts was calculated by using the formula:

$$\% \text{ Extractive yield} = \frac{\text{Weight of the extract}}{\text{Weight of the sample powder}} \times 100$$

Cell line and culture

The anti-cancer activity was studied on human breast cancer cell lines (MCF 7/MDA-MB-231), procured from National Centre for Cell Science, Pune, India. The cells were cultured in Dulbecco's minimum essential medium (DMEM) supplemented with, 10% heat inactivated Fetal bovine serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin-B (5 µg/ml) in a humidified atmosphere of 5% CO₂ at 37 °C until confluent. The cells were trypsinized with Trypsin-EDTA solution. The stock cultures were grown in 25 cm² flat bottles and the cytotoxicity studies were carried out in 96 well microtitre plates.

Screening for cytotoxicity by MTT assay against MCF-7 and MDA-MB-231

The MTT assay was performed as per the standard procedures [9]. MCF 7 Cells were plated in 96 well flat bottom microtitre plates at a density of 5 × 10⁴ cells per well and cultured at 37°C, in 5% CO₂ atmosphere, for 24 h, to allow cell adhesion. After 24 h, when a partial monolayer was formed, medium was removed and cells were treated with different concentrations of either the standard drug (Doxorubicin/Cisplatin) (0.5 µg/ml, 1.0 µg/ml, 2.5µg/ml, 5 µg/ml and 10 µg/ml) or the crude leaf extracts (31.25 µg/ml, 62.5 µg/ml, 125 µg/ml, 250 µg/ml and 500 µg/ml) for 48 h. After the treatment, the solutions in the wells were discarded and 50 µl of freshly prepared MTT (2 mg/ml PBS) was added to each well. The plates were shaken gently and incubated at 37°C in 5% CO₂ atmosphere for 3 h. The supernatant was removed and the formazan crystals formed in the cells were solubilized by addition of 50 µl of iso-propanol. The absorbance was read using a Microplate reader (Bio-Tek, ELX-800 MS) at 540 nm.

The percentage growth inhibition was calculated using the formula below:

$$\% \text{ Growth inhibition} = \frac{(\text{Control absorbance} - \text{test absorbance})}{(\text{Control absorbance})} \times 100$$

A graph was plotted with concentration (µg/ml) on x-axis and absorbance (540 nm) on y-axis for calculation of IC₅₀ values. IC₅₀ value is the concentration of drug required to kill 50% of cells in exponentially growing cultures after 48 h exposure to the drug.

The extract with least IC₅₀ value with MCF 7 was further tested using MDA-MB-231 breast cancer cell line and the IC₅₀ value was calculated as mentioned above.

Phytochemical screening

The phytochemical analysis, both qualitative and quantitative, was carried out according to standard procedures with the most effective extract, i.e., showing highest cytotoxic activity. Qualitative analysis was done to detect the presence/ absence of phytochemicals such as alkaloids by Wagner's test and Hager's test; flavonoids by alkaline reagent test and lead acetate test; tannins and phenols by ferric chloride test and gelatin test; Saponins by foam test; carbohydrates by Molisch's test and Benedict's test; amino acids by ninhydrin test and proteins by biuret test, glycosides by Keller Killiani test and bromine water test; steroids and terpenoids by salkowski's test and Liebermann Burchard's test according to the standard procedures [10].

Quantitative estimation of the phytoconstituents were done according to the standard procedures as follows: Total phenolic content by Folin Ciocalteu method [11], total flavonoids by Aluminium chloride colorimetric technique [12], alkaloids, tannins, saponins [13], proteins by Lowry method [14] and steroids / terpenoids by Liebermann-Burchard method [15].

Purification and structural elucidation of the cytotoxic principle

An attempt was made to purify and identify the cytotoxic principle from the effective extract by the following methods. MTT assay and phytochemical analysis was done to decide next step.

The crude extract was purified on TLC with chloroform: methanol: acetic acid (9:0.5:0.5 v/v) solvent system. The spots were visualized under UV at 254 nm. R_f values were calculated.

The extract was subjected to High Performance Liquid Chromatography (HPLC) with the HPLC-MS system (Thermo Finnigan Surveyor; Thermo LCQ Deca XP MAX) at IISc, Bangalore. The LC conditions of the analysis were as follows: mobile phase: Acetonitrile: Methanol (80:20 v/v); column: BDS HYPERSIL C18 (Reverse Phase) (4.6 mm × 150 mm × 5 µm); Detector: HPLC PDA / UV detector; wave length: UV @ 220nm-Channel A, 254nm for Channel B and 290nm for Channel C; flow rate: 0.4 ml/min; Temperature: ambient; injection volume: 10 µl; Run time: 40 min; Software : Xcalibur. The mass spectrometer operated in positive mode from a range of 50 m/z to 600 m/z with a source voltage of 4.5kV. Sample Tray Temp: 15°C, Column oven temp: 30°C, Capillary Temp (C): 320, Capillary voltage (V): 16.00,

Nebulization gas flow: Helium at 1 mL/min approx, was maintained at 0.1Pa (10^{-3}) in the mass analyser cavity.

The spot was subjected to column chromatography on a silica gel column (17 x 1 cm). Elution was done with acetonitrile: methanol (80:20% v/v) at a flow rate of 0.4 ml/min and 8 fractions were collected (4 ml each). The eluant fractions were analysed qualitatively for steroids and terpenoids by Liebermann-Burchard reaction. The fractions positive for steroids/ terpenoids were pooled together; evaporated to dryness and tested for cytotoxicity by MTT assay against MDA-MB-231 breast cancer cell line.

Column collected fraction was subjected to TLC using Silica gel plates (Merck, Germany) and toluene: ethylacetate (7:3 v/v) as solvent system. The spots were visualized under UV at 254 nm. R_f values were calculated. The spots were scraped and tested for cytotoxicity by MTT assay against MDA-MB-231 breast cancer cell line.

Statistical analysis

The experiments were done in triplicates ($n=3$) and the values expressed as mean \pm standard deviation values. Analysis was

done using one-way ANOVA followed by LSD posthoc test for comparison between control and treatment groups using SPSS version 14 software (IBM). $P < 0.05$ was considered to be statistically significant.

Results and discussion

The leaves of *T. procumbens* L. were dried and extracted with chloroform, ethanol and methanol. The crude extracts of *T. procumbens* L. in methanol, ethanol and chloroform were called as TPM, TPE and TPC respectively. The yield of the crude extract was highest in TPM (11% w/w) followed by TPE (8% w/w) and TPC had the least (1.56% w/w). In the preliminary screening of the extracts with MCF-7 breast cancer cell line (estrogen receptor positive and from breast tissue), the chloroform soluble crude extract (TPC), has shown effective cytotoxicity (IC_{50} : 136 ± 2.1 $\mu\text{g/ml}$), ($p < 0.001$) in comparison with the methanol (IC_{50} : 190 ± 2.2 $\mu\text{g/ml}$) and ethanol (IC_{50} : 248 ± 3.2 $\mu\text{g/ml}$) extracts (Figure 1). The IC_{50} value of doxorubicin (positive control) was 1.09 ± 0.03 $\mu\text{g/ml}$ (Figure 1).

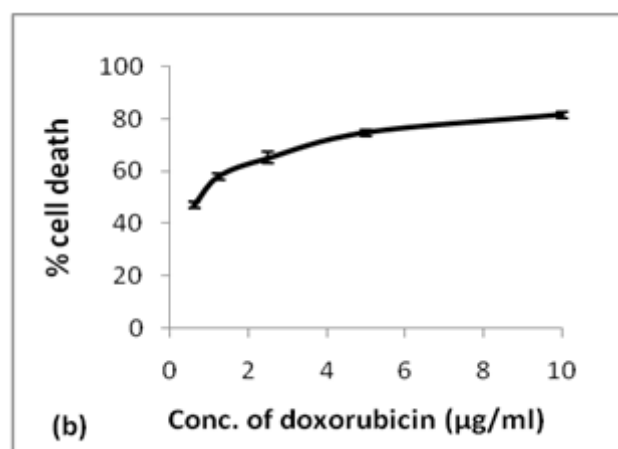
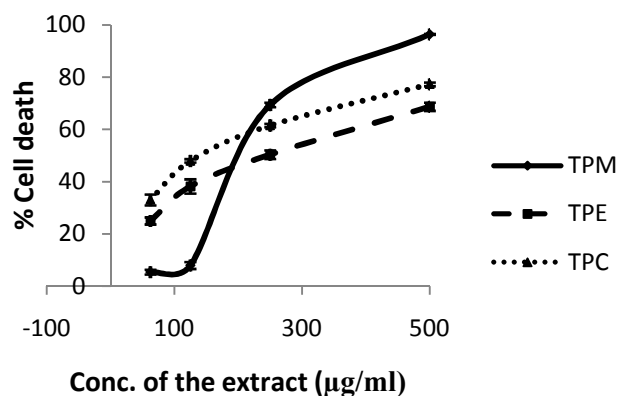


Figure 1. Effect of (a) *T. procumbens* crude extracts and (b) Doxorubicin (positive control) on MCF -7 cell line by MTT assay. The effect of the crude extracts were tested at concentrations from 31.25 $\mu\text{g/ml}$, 62.5 $\mu\text{g/ml}$, 125 $\mu\text{g/ml}$, 250 $\mu\text{g/ml}$ and 500 $\mu\text{g/ml}$. Positive control doxorubicin was tested at concentrations from 0.62 $\mu\text{g/ml}$, 1.25 $\mu\text{g/ml}$, 2.5 $\mu\text{g/ml}$, 5 $\mu\text{g/ml}$ and 10 $\mu\text{g/ml}$; TPM: methanol soluble extract; TPE: ethanol soluble extract; TPC: chloroform soluble extract.

In spite of the low yield of the chloroform soluble extract it had highest cytotoxic potential in comparison with the ethanol and methanol soluble extracts. The effective extract, i.e., TPC was then tested in MDA-MB-231 cell line, a triple receptor negative breast cancer cell line of metastatic origin. The IC_{50} value of TPC against MDA-MB-231 breast cancer cell line was 129.1 ± 2.3 $\mu\text{g/ml}$ ($p < 0.001$) and that of cisplatin (positive control) was 0.5 ± 0.13 $\mu\text{g/ml}$. There was no significant difference ($p > 0.05$) between the IC_{50} values of the extract against both the cell lines (Table 1). Earlier, cytotoxicity of aqueous and acetone, leaf and flower extracts of *T.*

procumbens was reported by Vishnupriya *et al.*, but against prostate epithelial cancer (PC3) cell line [7, 8]. Although they have not mentioned the IC_{50} value, 93% cell death has been reported at a concentration of 250 $\mu\text{g/ml}$ with the acetone leaf extracts and 82.28% cell death with the acetone flower extracts [7, 8]. In the present study the chloroform soluble extract showed an IC_{50} value of 129.6 $\mu\text{g/ml}$. The differences in the effectiveness reported in the studies reported by Vishnupriya *et al.*, and the present study could be because of the phytochemical composition of the extracts. The phytochemical concentration is known to vary not only with the

solvent used but also with the method of extraction [16]. TPC of this study was prepared by maceration with constant shaking while the aqueous and acetone extracts used by Vishnupriya *et al.*, was

soxhlet distilled [7, 8]. Heat labile phytochemicals can be lost during Soxhlet distillation [17].

Table 1. Comparison of IC₅₀ values of TPC crude extracts in MCF-7 and MDA-MB-231 breast carcinoma cell line

Name of the extract	IC ₅₀ value (µg/ml)		P value
	MCF-7	MDA-MB-231	
TPC	136.0 ± 2.1	129.0 ± 2.3	0.63 (NS)
Positive control	1.09 ± 0.3	0.5 ± 0.13	-

The IC₅₀ values of the crude extracts against MCF-7 and MDA-MB-231 breast carcinoma cell lines were compared from the graphs plotted with concentration of extracts (µg/ml) on the X axis and cell death (%) observed on the Y axis and expressed as µg/ml. TPC: chloroform soluble extract. p > 0.05, NS: statistically no significant difference.

Qualitative analysis of TPC revealed the presence of only steroids and terpenoids (Table 2) at a concentration of 77.4 ± 0.3% w/w by

Liebermann-Burchard method. The other phytochemicals if present were probably in undetectable quantities.

Table 2. Phytochemical composition of the *T. procumbens* chloroform soluble crude extracts

Phyto-constituents	TPC	
	Qualitative	Quantitative (% w/w)
Flavonoids	-	-
Alkaloids	-	15.33 ± 0.6
Phenols	-	5.19 ± 0.2
Tannins	-	3.43 ± 0.0
Saponins	-	19.33 ± 0.1
Carbohydrates	-	-
Glycosides	-	-
Proteins	-	-
Steroids/ Terpenoids	+	77.40 ± 0.3

'+' Presence of phytochemical '-' absence of phytochemical

Qualitative analysis of the chloroform extract of the leaves of *T. procumbens* have been reported by various groups earlier as well [18]. However they have prepared the extract by soaking 400 g of the leaf powder in 500 ml chloroform in cold condition with occasional shaking while the extracts of this study was 10%w/v. Their qualitative analysis revealed the presence of steroids, saponins, alkaloids, flavonoids and diterpenes. The difference between the present study and the study by Sawant and Godghate is the temperature of extraction and also the solute: solvent ratio. Earlier, reports have shown that temperature maintained during extraction protocols has an effect on the phytochemical composition of the extract [17, 19].

However the quantitative estimation of chloroform soluble crude extracts of *T. procumbens* did show the presence of phenols, alkaloids, saponins and tannins, but at very low concentrations of 5.19% w/w, 15.33% w/w, 19.33% w/w and 3.43% w/w, respectively, in comparison with that of steroids (77.4% w/w) (Table 2).

Thin layer chromatography of the chloroform soluble crude extract using chloroform: methanol: acetic acid (9:0.5:0.5 v/v) solvent system showed a single spot with R_f value 0.66 suggesting the

presence of either a single compound or a group of compounds with similar polarity. However, in an attempt to isolate the bioactive principle from the leaves of *Tridax procumbens*, Sankarnarayan *et al.*, had also used TLC of methanol extract of *Tridax procumbens* partitioned with ethyl acetate and water [20]. The ethyl acetate fraction yielded four compounds when passed through a silica gel column with chloroform: methanol solvent system. Of the four Compounds, the one with R_f value 0.66 showed anticancer activity. Further identification revealed the compound to be lupeol, a triterpenoid with molecular weight 426.7. The compound of the present study although a steroid/ triterpene, may or may not be lupeol since the solvent of the extract and the solvent system used for TLC are different.

HPLC-MS of the TPC extract revealed the presence of 5 peaks (Figure 2 and Table 3). A major peak (57.59% concentration) was obtained at a retention time of 4.78 min and MS data of this peak revealed presence of two fragments of molecular weight 475.80 and 701.80. Database search for phytochemical with molecular weight 475.80 and 701.80 suggested that the compounds could be 3β, 9β-Dihydroxy-18 -oleanan-28-oic acid (1, Figure 3), a derivative



of oleanolic acid and β , 28-Bis (cinnamoyl) betulin (2, Figure 3) respectively, both from the triterpenoid family. The significant

activity in the chloroform soluble extract may be attributed to the presence of above mentioned compounds [21].

Table 3. HPLC-MS of TPC with R_t values

Retention time (R_t)	% Area	% Height
4.78	59.52	57.59
12.14	3.63	6.71
14.29	8.19	12.49
27.08	10.60	9.57
31.70	18.06	13.63

HPLC-MS of the TPC extract with Retention time, % Area and % height.

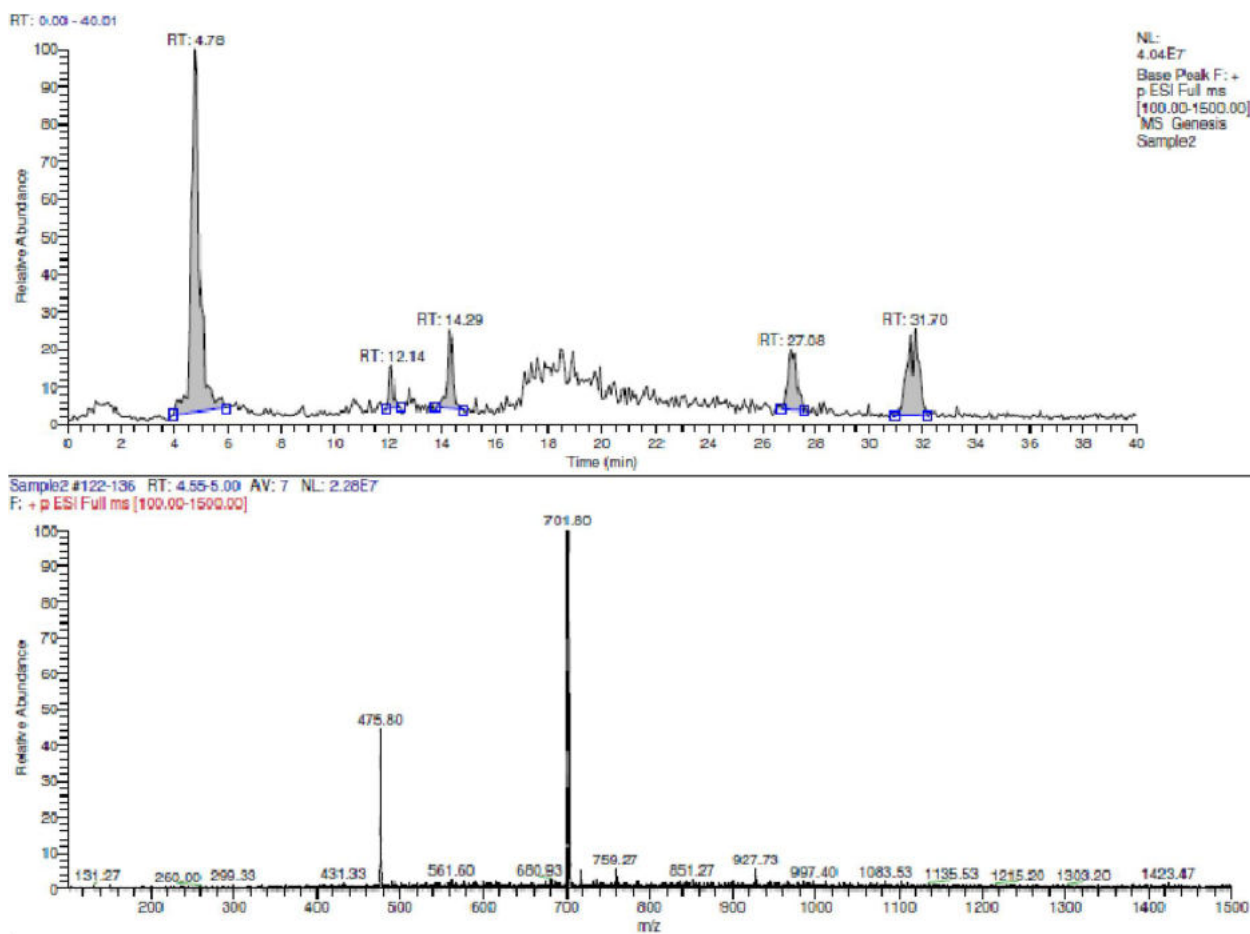


Figure 2. HPLC-MS chromatogram of the chloroform soluble crude extract of *Tridax procumbens* L. Chromatographic conditions: Mobile phase: acetonitrile: methanol (80:20 v/v), BDS HYPERSIL C18 (Reverse Phase) (4.6 mm \times 150 mm \times 5 μ m); Detector: HPLC PDA / UV detector; Wave length: UV @ 220nm-Channel A, 254nm for Channel B and 290nm for Channel C; Flow rate: 0.4 ml/min; Temperature: ambient; injection volume: 10 μ l; Run time: 40 min; Software: Xcalibur.

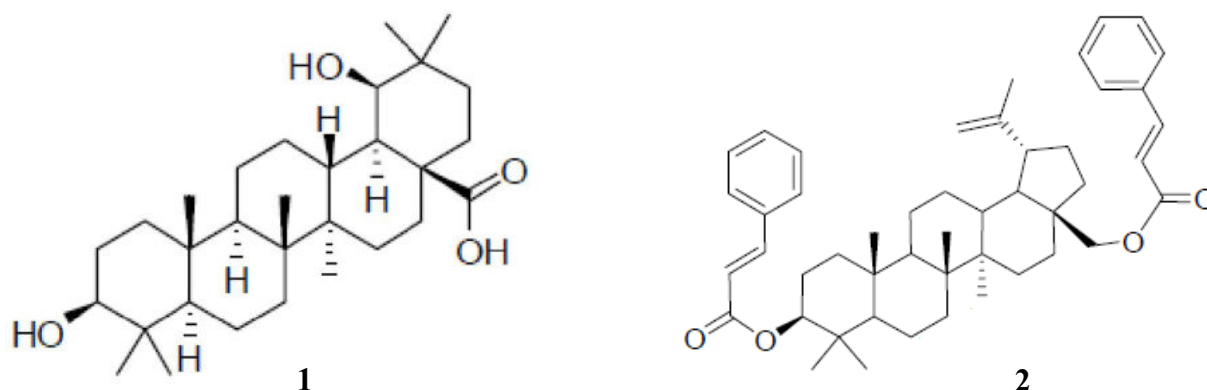


Figure 3. Structures of terpenoids, 3 β , 9 β -Dihydroxy-18-oleanan-28-oic acid and 3 β , 28-Bis(cinnamoyl) betulin (1 and 2).

Column chromatography of TPC eluted with acetonitrile: methanol (80:20 v/v) solvent system yielded 8 fractions. The eluants were tested for the presence of steroids and triterpenoids and the positive fractions were pooled together and evaporated to dryness. The pooled fraction showed effective cytotoxicity against MDA-MB-231 cell line at an IC_{50} value of $84.7 \pm 0.9 \mu\text{g/ml}$ by MTT assay (Table 4). On TLC profiling, two bands with R_f value 0.96 and 0.89 were obtained. The band with R_f value 0.96 exhibited effective

cytotoxicity against MDA-MB-231 cell line (IC_{50} : $57.5 \pm 1.3 \mu\text{g/ml}$) while the spot with R_f value 0.89 was not cytotoxic. This correlates with the HPLC-MS result where two fragments were obtained. With the activity guided isolation/fractionation, the IC_{50} value of the extract (TPC) decreased from $129.1 \pm 2.3 \mu\text{g/ml}$ to $57.5 \pm 1.3 \mu\text{g/ml}$ against MDA-MB-231 cells by MTT assay indicating the increase in the cytotoxicity (Table 4).

Table 4. IC_{50} values of the activity guided fractions of *T. procumbens* L. against MDA-MB-231 cells

Activity guided fractions	IC_{50} value ($\mu\text{g/ml}$)	Conc. of steroids & terpenoids (% w/w)
Crude extract (TPC)	129.1 ± 2.3	77.4 ± 0.25
Column collected fraction	84.7 ± 0.9	82.4 ± 3.26
TLC scraped spot 1	57.5 ± 1.3	91.8 ± 4.12
TLC scraped spot 2	No toxicity	ND

Column collected fraction and TLC scraped spots were screened against MDA-MB-231 cell line at 5 different concentrations (6.25 $\mu\text{g/ml}$, 12.5 $\mu\text{g/ml}$, 25 $\mu\text{g/ml}$, 50 $\mu\text{g/ml}$ and 100 $\mu\text{g/ml}$) and the % cell death was calculated. Graphs were plotted with concentration of extracts ($\mu\text{g/ml}$) on the X axis and cell death (%) observed on the Y axis. The IC_{50} values were determined from the graph and expressed as $\mu\text{g/ml}$. ND: Not determined.

Oleanolic acid, a pentacyclic triterpenoid, widely distributed in food and herbal medicines is known to exhibit cytotoxic effects through various molecular targets. Oleanolic acid is shown to reduce the invasion and migration capacity in the U-87MG glioma cells by inhibiting the mitogen activated protein kinase /extracellular signal-regulated kinase (MAPK/ERK) pathway [22]. Antiproliferative action of oleanolic acid in MCF-7 breast cancer cells has been demonstrated to be by cell cycle arrest [23, 24]. On the other hand derivatives of oleanolic acid are known to be more cytotoxic, and proapoptotic against breast cancer [25].

Betulin is reported to be non-cytotoxic compound, although oxidized derivatives of betulin like betulinic acid have been reported to have anticancer activity [26]. However, treatment of cinnamoyl betulin at a concentration of 8 μM on HL cells and 50 μM on Huh-7 cells showed no cell death, indicating that the compound is non-cytotoxic [27]. Use of terpenoids with potential for anticancer activity has been very well established. *T. procumbens* is found to

be rich in terpenoids such as bis-bithiophene, taraxasteryl acetate, beta-amyrenone, lupeol, betulinic acid and oleanolic acid [28, 29]. The steroids and terpenoids are reported for their cytotoxic activity against various cancer cell lines through apoptosis, through regulation of various transcription and growth factors as well as intracellular signaling mechanisms [30]. In the present study, the cytotoxicity of the effective extract was also tested against triple negative breast cancer cell line MDA-MB-231. The effectiveness of TPC suggests that the cell death may be due to a mechanism which is common in both these cell lines. Manjamalai *et al.*, have also attempted to study the mechanism and concluded the cell death may be due to apoptosis [31].

Conclusion

In conclusion, the cell viability inhibition in MCF-7 and MDA-MB-231 cells of this study may be attributed to the presence of oleanolic acid derivative, in the chloroform soluble extract of *Tridax*

procumbens L. and this finding validates the basis for its use in traditional medicine.

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Conflict of Interest

The authors declare no conflict of interest.

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