ISOLATION AND CHARACTERIZATION OF PHYTOCHEMICAL CONSTITUENTS OF CLEOME RUTIDOSPERMA LEAF METHANOL EXTRACT AND ITS CYTOTOXICITY AGAINST MCF-7 CELLS

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ABSTRACT

Natural resources like medicinal plants have been used traditionally to treat or manage various diseases and disorders. Cleome species possess to have major bioactive compounds with clinical significance. The present study was carried out to exploit the *Cleome rutidosperma* for its phytoconstituents and to isolate major bioactive compounds and their efficacy as cytotoxic agent against MCF-7 breast cancer cells. The isolated compound was characterized with GC-MS spectroscopy and the structure was elucidated using spectral data analysis which revealed the presence of a cardiac glycosides derivative that was isolated for the first time from *Cleome rutidosperma* namely Acetic acid 3, 14-dihydroxy-10,13-dimethyl-17-(6-oxo-6H-pyran-3-yl)-hexadecahydro-cyclopenta[α]phenanthren-16-yl ester (Bufotalin). The crude and isolated Bufotalin possess to have potential anticancer activity with an IC_{50} value of 22.86 µg/ml and 3.895µg/ml respectively, against the breast cancer cell lines. The plant-mediated isolated cardiac glycoside bufotalin will be a potential agent for the control of the proliferation of cancer cells.

Keywords: Bufotalin, GC-MS, FTIR, NMR, MCF-7, *Cleome rutidosperma*.

Introduction

Nature is the best integrative chemist that is of great aid to humankind in many ways. The most abundant healthy natural resources of life are plants[1]. Medicinal plants play a crucial role in the discovery of drugs. The origin of modern medicines is highly dependent on traditional medicines[2]. Ayurveda is a restorative framework fundamentally honored in India that has been existent for 5000 years [3]. The world health organization has estimated that more than 80% of the global population relies on medicinal plants for their primary healthcare[4]. These plants have the natural ability to produce a plant metabolite to balance and survive the harshening environmental conditions and microbial attacks[5]. In recent years, plant species have been scientifically evaluated for their possible medicinal applications. Herbal medicines are a rich source of bioactive compounds that are safe and environmentally friendly[6].

Plants are the natural reservoirs of bioactive secondary metabolites. Alkaloids, tannins, flavonoids, and phenolics are the most important bioactive constituents present in medicinal plants[7]. The chemical constituents and pharmacological properties of the vast wealth of endangered species were not completely explored. Identification and evaluation of the presence of active principles in these plants is the need of the hour. Every year more than 8 lakh cancer patients were diagnosed in India, while the breast cancer and cervical cancer were found to occur in majority of women across the world8. Breast cancer is the most prevalent leading cause of cancer-related death in women[9]. There were about 1,45,000 new cases in India in 2012 and about 70,218 patients died due to breast cancer during this period. It is estimated that the number of cases will increase by 70% in 2035[10]. The incidence of breast cancer is higher in urban μ which can be probably due to the change in lifestyle and environmental factors. Traditional medicinal plants are the conventional sources for the separation and isolation of phytoconstituents for the treatment of cancer-related disorders[11, 12]. Novel therapeutics with potential anticancer activity must be developed to prevent cancer-related deaths, since chemotherapy, and radiation treatments lead to severe side effects. The United States National Cancer Institute has recognized the potential of natural products of plant material as chemo-protective material[13].

Cleome rutidosperma, commonly known as “Fringed Spider Flower” which belongs to the family Cleomaceae, is a small herb that grows up to 70 cm in height with trifoliate leaves, and small violet-blue flowers. This plant is native to West Africa and has now become naturalized in the tropical and temperate regions of Southeast Asia and other parts of the World[14]. The medicinal properties of *C. rutidosperma* has been reported as antimicrobial activity, antioxidant and free radical scavenging activities[15]; anti-diabetic effect[16]; anti-

hyperglycemic effect[17]; antifungal activity[18]; and anti-inflammatory effect[19]. *Cleome rutidosperma* belonging to the family Cleomaceae comprises 14 genera with two hundred species and most of the species present in the genus were reported to possess bioactive compounds with anticancer potential[19, 20]. Despite their clinical significance, most of the species belonging to the cleome family were not exploited for their specific chemical constituents. Thus, the earlier studies have established the significant biological activities of *Cleome rutidosperma* on crude preparations. In the present study, an attempt has been made to isolate and elucidate the structure of the isolated compound and to evaluate its efficacy against human cancer cells.

METHODS

Chemicals and media

Chemicals for extraction and column chromatography (analytical grade), methanol was purchased from Hi-Media, Mumbai, India. Silica gel (60-120 mesh) was used for column chromatography and pre-coated silica gel 60 GF254 plates were used for Thin Layer Chromatography (TLC) from Merck Limited, Germany.

Plant material

The whole plant *Cleome rutidosperma* was collected and a voucher specimen was preserved for verification in the Plant Biology and Biotechnology Department, Presidency College, Chennai, Tamil Nadu, India. The disease-free leaves were separated and washed thoroughly with tap water followed by rinsing in double-distilled water and shade dried for fifteen days.

Preparation of extract

Cleome rutidosperma dried powder was used for the hot extraction process with Soxhlet apparatus where methanol is the solvent, the process was continued for eight hours and the collected extracts were concentrated using a vacuum evaporator.

Isolation of bioactive compound Activated silica gel (60-120 mesh) was packed into a glass column (120x30 mm) with n-hexane as solvent was used for the column chromatography[21]. 3grams of methanol extract was loaded on silica gel for the separation of components by the wet slurry method. The column was eluted with the mixtures of hexane and ethyl acetate and finally with 100ml of 100% methanol. The separated fraction was evaluated with TLC using hexane: ethyl acetate: methanol (1.2: 0.5: 0.3).

Spectroscopic analyses of bioactive compound

Gas Chromatography and Mass Spectrogram (GC-MS)

The separated active compound was subjected to gas chromatography-mass spectrometry (GC-MS) analysis which was conducted in JEOL GC mate equipped with Elite- 1 capillary column (30m x 0.25mm) coupled with turbo mass. The column temperature was 110 ºC initially held for 2 min, then programmed to 280 ºC at a rate of 5 ºC/min in the split mode. Helium was used as a carrier gas with a flow rate of 1ml/min. The injector temperature was at 250 ºC. The Mass spectrum observed was referred to by the library National Institute of Standards and Technology (NIST).

Fourier Transform Infrared Spectroscopy (FTIR)

50 mg of the column-separated compound was dissolved in 25 ml of deuterated methanol. The sample was dispersed in KBr pellets and loaded in the sample holder of the Perkin Elmer spectrum FT-IR instrument and operated in the range of 450-4000 cm^{-1}. The functional groups were detected from the obtained spectral data.

Nuclear Magnetic Resonance (NMR) Spectroscopy

20mg of the isolated compound was analyzed by NMR (^{1}H and ^{13}C) by dissolving the compound in 0.5 ml of deuterated methanol and the spectral data was recorded on a Bruker AVANCE III 500 MHz for 6 h at room temperature. 5 mm PABBO-BB probe was used. Methanol-d (Me OD) was used as a solvent. The chemical shifts (δ) were expressed in ppm. The region for ^{1}H NMR from 0 to 10 ppm and 0 to 200 ppm for ^{13}C NMR was applied for scanning.

IN VITRO ANTICANCER STUDIES

CYTOTOXICITY ASSAY

Cytotoxic efficacy of methanol mediated crude leaf extract of Cleome rutidosperma and the derived compound of the extracts were evaluated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The cells were seeded in 96 well microtiter plates with Dulbecco's Modified Eagle Medium (DMEM) and 10 % Fetal bovine serum (FBS) and incubated till the confluency of 90 to 95% was achieved. After the period of incubation, the wells were loaded with serum-free media and incubated for 60 minutes. Further, the wells were treated with different concentrations of crude methanol extract and the isolated compound Bufotalin and incubated for 24 hours at 37 °C, after the period of incubation of 20 microliters of 5mg/ml (Working solution), MTT was added and further

incubated for 4 hours. At the end of incubation, the medium was removed carefully, and Formazan crystals formed were solubilized using 100 microliters of Dimethyl sulfoxide (DMSO). After 5 minutes the optical density was measured with a microplate reader at 570nm. The growth inhibition (IC_{50}) was determined from the percentage of cell survival which was calculated as per the below equation:

Cell Viability (%) = Absorbance of Control X Absorbance of Test X 100

MCF -7 cells were treated with an isolated compound using 4'-6-Diamidino 2-phenylindole (DAPI) nuclear staining to observe the apoptotic morphology of the cells. The cells were treated with IC_{50} concentration of Bufotalin for 24 hours and the cells were washed with phosphate buffer saline (PBS) and stained with 5µl of DAPI. The Nuclear morphology was examined under fluorescent microscopy. The apoptotic morphology of the cells was studied by staining the cells with Acridine
orange and Ethidium bromide and the cells were observed under fluorescence microscopy.

Statistical analysis

Data were expressed in Mean ± Standard Error (S. E). Statistical analysis was done using a statistical package for social sciences (SPSS) software.

RESULTS

Medicinal plants are the source of bioactive compounds and the qualitative phytochemical findings of the methanol solvent-mediated crude leaf extract of *Cleome rutidosperma* reveal the presence of alkaloids, flavonoids, cardiac glycosides, phenols, steroids, etc., Compounds were isolated from the methanol solvent extract of the plant and the extracts were subjected to column chromatography for the separation of the bioactive compound using n-hexane and ethyl acetate as solvents. Six fractions like fraction A (65:35), fraction B (60:40), fraction C (30:70), fraction D (20:80), fraction E (10:90), and fraction F (5:95) were observed in the eluted column of which fraction F showed prominent antimicrobial activity when compared to other fractions. Further purification of this selected fraction has shown a single spot-on TLC (Fig. 1). The GC of the isolated bioactive compound presented in Figures 2 shows the retention time in the column and the detected peaks. The mass spectrometer analysis reveals the presence of a compound 3, 9 á; 14, 15-Diepoxypregn-16-en-20-one, 3, 11 á, 18-triacetoxy- (Fig. 2).

FT-IR spectrum of the purified compound is shown in table 3. It exhibited absorption at 3418, 2053, 1637, 1384, 1323, 1145, 1101 and 621 cm^{-1}. The peak at 3418 cm^{-1} shows the O-H stretch which may be due to the presence of alcohol. The band at 2053 cm^{-1} represents the C=C group. The peak at 1637 cm^{-1} corresponds to C=O stretch, confirming the presence of the unsaturated carbonyl group/ aliphatic group. The bands at 1384 and 1323 cm^{-1} represent the out of plane C-H def -vibration. The peaks at 1145 and 1101 cm^{-1} show C-O stretch which indicates the presence of 3º alcohols and 2º alcohols respectively. In addition, the peak at 621 cm^{-1} indicates a C-H bend which may be due to the presence of an alkene group or methylene rocking.

The column eluted purified compound the yellow crystal, $C_{26}H_{36}O_6$, molecular weight (MW) 444.56.[1] H NMR (500 MHz, MeOD):0.80 (s, 3H), δ 1.21(t,2H),1.21 (t,2H),1.51 (t,1 H),1.51 (q,2H),1.61(q,2H),1.97 (q,1H),2.05 (m,1H),2.06 (t,2H),2.15 (q,2H),2.23 (d,2H),2.68 (d, 1H),4.26 (m,1 H),4.51 (broad,s) and 5.24 (q, 1H) were considered as tetra cyclic hydrocarbon ring. Pyran ring were observed at 2.35 (s, 3H), 5.69 (d, 1H),7.53 (s, 1H) and 7.62 (d, 1H) as acetyl group (Fig.3).

^{13}C NMR was performed to observe the presence of bufotalin and the characteristic proton signal were observed at (125 MHz, MeOD):δ 9.88 (C-19),9.90 (C-18),22.32 (C-2"),22.35 (C-11),24.62 (C-7),29.30 (C-6),47.09 (C-2),47.26 (C-1), 47.43 (C-12),47.50 (C-15),47.55 (C-4),47.57 (C-10),47.73 (C-5), 47.73 (C-8),47.74 (C-13),47.77 (C-9),47.77(C-17), 47.90 (C-16), 47.94 (C-3), 48.11 (C-14),102.24 (C-5^1),105.55 (C-2^1),110.24 (C-3^1),110.42 (C-4^1),171.63 (6 (C=O)) (Table 2 and Fig.4).

The structure of the isolated compound was elucidated using ^1H NMR and ^{13}C NMR and the molecular formula was found to be $C_{26}H_{36}O$. The m/z value of the isolated compound, according to MS data was 429.18 (M^+-CH_3), 401.06 (M^+-$COCH_3$), 355.10 (M^+- $COCH_3$-CO_2-H_2), 281.07 (M^+-$C_7H_{15}O_4$), 221.11 ($C_{15}H_{24}O^+$) 182.76 ($C_{12}H_{22}O^+$), 147.05 ($C_{10}H_{11}O^+$), 73.05 ($C_4H_9O^+$). Based on ^1H and ^{13}C NMR, this compound contains 19 Tetracyclic Hydrocarbon rings, five pyran rings, and two acetyl groups which were named Bufotalin (Acetic acid 3, 14-dihydroxy-10, 13-dimethyl-17-(6-oxo-6H-pyran-3-yl)-hexadecahydro-cyclopenta[α] phenanthren-16-ylester) (fig.5).

Cytotoxic activity of methanol crude extract and isolated compound Bufotalin

Methanol extract of *Cleome rutidosperma* was tested against MCF-7 breast carcinoma cell line with varying concentrations of 25µg/ml, 50µg/ml, 75µg/ml, 100µg/ml, and 125µg/ml.

the methanol extract exhibits significant activity against the breast cancer cell line with an IC_{50} value at 22.86µg/ml after 48hrs (Table3). The Cytotoxicity of the isolated compound was evaluated against MCF-7 breast cancer cell lines using an MTT assay and the cells were treated with different doses of isolated compound bufotalin ranging from 0.0044µm/L to 0.0179µM/L. The results reveal the potential of the Bufotalin against the breast cancer cell lines with an IC_{50} value of 2.68µg/ml. Further, the apoptosis in the anticancer activity of Bufotalin was observed using DAPI and Acridine orange and Ethidium bromide staining to observe the nuclear and cell morphology. The DAPI results reveal that the impact of Bufotalin over the MCF cells results in the chromatin condensation resulting in the bright blue colour of the apoptotic cell nuclei and the live cells were light blue. The apoptotic cell percentage increases with increasing concentration and confirms the anticancer efficacy of the Bufotalin isolated from the crude methanol leaf extract of *Cleome rutidosperma*.

The data presented in Table 4 represents the cell viability assay using the MTT assay to check the efficacy of the isolated compound Bufotalin from the methanol leaf extract of *Cleome rutidosperma* against MCF-7 breast carcinoma cell line with the concentration ranging from 2µg/ml, 4µg/ml, 6µg/ml, 8µg/ml and 10µg/ml. The cell lines were maintained in the structural environment, and the cytotoxic activity was observed at 24hrs and 48hrs after the exposure as that of crude extracts. The potential compound Bufotalin shows a potential anticancer efficacy against the MCF-7 cell line with an IC_{50} value of 12.84 after 24hrs and 3.895µg/ml after 48hrs.

The enlargement of cells with membrane blebbing was apparent in the cells. The morphological observation (Figure 6b and d) reveals the structural alterations in the shape of the cells, apoptotic cells with vacuolation are evidenced and the cells were detached from the monolayer culture suggesting cell death.

DISCUSSION

Plants produce a diverse range of bioactive molecules making them a rich source of different types of medicines. The natural extracts of plants are an important source for the identification of new biologically active compounds with possible applications in the pharmaceutical field. Phytotherapy embraces the isolation from herbs, of compounds with unique chemical structures, which are pharmacologically active[22]. The bioactive compounds were characterized using chromatographic methods and crude methanol leaf extract of *C.*

rutidosperma was purified by silica gel column chromatography. Six fractions were collected from the column of which fraction F shows prominent antimicrobial activity. To identify the nature and structure of the compound, it was further subjected to FTIR, ^{1}H NMR, and ^{13}C NMR. ^{1}H NMR and ^{13}C NMR and observations reveal the presence of the carbonyl group, alcohols, and aliphatic groups.

NMR spectral analysis of the isolated compound confirms the structural configuration and the compound materials have been identified as Bufotalin. Bufotalin represents the class of compounds that belongs to cardiac glycosides. The presence of bufadienolides was recorded from plant and animal sources[23]. The bufadienolides were characterized by six membrane lactone rings with 2 double bonds attached in 17 beta position at the positive Hexa hydro cyclo Penta phenanthrene skeleton.

In Chinese medicine, bufadienolides were commonly used to treat various disorders, especially in cancer patients in China[24]. Bufotalin exhibits many pharmacological and biological activities in addition to antitumor and cardiotonic activity[25]. Anti-cancer agents induce apoptosis to arrest the tumor cell proliferation and progression[26]. Bufalin is shown to induce cell differentiation and apoptosis were interlinked and regulated by distinct protein kinase C isoenzyme[27]. Cardiac glycosides like bufotalin act as potent inhibitors over cancer cell growth[28]. Cardiac glycosides inhibit cancer cell proliferation even in minimal concentrations[29]. Bufotalin was used in traditional Chinese medicine to inhibit the growth of the proliferation of human hepatocarcinoma cells Hep-G2[30]. Bufotalin is a potential candidate for the treatment of pancreatic cancer when administered along with gemcitabine a standard anticancer drug[31].

Two alkaloids aspara doxonine and para doxenoline were isolated from the chloroform fraction of methanol extract of *C.paradoxa*[32]. A steroid derivative 17-(4-hydroxy-1, 5-dimethylhexyl)-2, 3, 7-(acetyloxy) gona-1, 3, 5(10)-trien-15-ol was isolated and identified from the phenolic extract of *C.arabicais* and named as Clive-92[33,34]. Ten compounds were identified by 35 Jordheim *et al.* (2009) from the flowers of *C.hassleriana*. Palmitic acid, Cleomaldeic acid, glucocapparin, glucocleomin were isolated and identified from *C.viscosa*[36,37]. In the present investigation a cardiac glycosides derivative was primarily isolated from *Cleome rutidosperma* namely Acetic acid3,14-dihydroxy-10,13-dimethyl-17-(6-oxo-6H-pyran-3-yl)-hexadecahydro-cyclopenta[α]phenanthren-16-yl ester.

Alkaloids, polyphenols phenylpropanoids, and terpenoids were said to possess anticancer properties.[38,39]. NCI- United States of America has set the limit for the activity for crude extracts with the IC_{50} value i.e., 50% of inhibition of proliferation with less than 30µg/ml after the exposure to 72 hrs. of exposure[40]. The crude extract with the IC_{50} value of less than 20µg/ml is highly cytotoxic in nature[41]. The results observed in the present study show the potent cytotoxic effect on MCF-7cells with methanol and aqueous extract of *Cleome rutidosperma*. The IC_{50}value was 108.260µg/ml concerning 24hrs and 22.86µg/ml after 48hrs of exposure. The IC_{50} value was much lower than the recommendation of NCI, the USA for the characterization of a pure compound as the anti-carcinogenic agent.

The anti-cancer sensitivity of *E. guineermisis* is due to the presence of flavonoids[42], phytocompounds alkaloids, and flavonoids of *Onobis hirta* are responsible for the superior anti-cancer activity against cancer cells[43]. Polyphenols and flavonoids are known to have antioxidant and anticancer activity[44]. As the prevalence of cancer cases is on the rise, it is necessary to screen and search the naturally available bioactive compounds with the potential to act against the cancer cells. Many compounds have been analyzed for their anticancer potential. Out of 974 small molecules screened from 1981 to 2006, 12% are designed from natural products inhibitor of the target of interest, and inhibition of active sites of the endogenous substrates such as ATP[45].

Bufadienolides are an important group of polyhydroxy C-24 steroids and their glycosides. Bufadienolides were derived both from animal and plant sources. The plant families Crassulaceae and Hyacinthaceae are abundant sources of bufadienolides, a type of cardiac glycoside traditionally used for the treatment of cardiac dysfunction and show some toxic effects[46]. The structural modifications of these compounds may make them a productive area of research and to design of a new lead compound. The compound that has the potential to kill the cancer cells with a specific concentration while not affecting the non-malignant cells was therapeutically useful. The cardiovascular side effect of these compounds pronounced restriction in the application of Bufotalin for the treatment of cancer in the form of chemotherapeutic drugs.

In the present study, it is evident that the nontoxic compound bufotalin was isolated from the methanol leaf extract of *Cleome rutidosperma*. Many researchers are in the search of novel compounds with specific anticancer potential. The ethnomedicine importance of bufotalin intended to study the cytotoxic efficacy of the plant-mediated compound isolated from

Cleome against the MCF-7 cell line. The result presented in the table reveals the cytotoxic effects of bufotalin on breast cancer cell lines. With the IC_{50} value 12.840µg/ml after 24hrs and 3.893µg/ml with reference to 48hrs, the results suggest the cytotoxicity of bufotalin on cancer cell lines.

The cytotoxic effect of bufotalin based on the time and dosage has been reported against Hep 3b, HT-29, and MCF-7 cells[47]. It is evident from the results that bufotalin has time-dependent activity concerning MCF-7 cell lines, further the anticancer potential of bufotalin on a nude mouse model suggesting that the bufotalin inhibits the cancer growth by the activation of p53 signaling[48]. The growth inhibitors activities of the drugs utilized to treat cancer cells were achieved by the induction of apoptosis[49]. The present study reveals a dose-dependent increase in the percentage of apoptosis which amounts to 69.65% proved to 10µg/ml concentration when compared to control.

Bufadienolides were considered a potent cytotoxic agent against different cancer cells[50]. Chemically modified bufadienolides can kill the malignant cells without damaging the normal non-malignant cells[51]. Biotransformation is a process by which the nature of the substances was altered by the secreting metabolites; structural diversity plays a major role in the mechanism of action against the Cancer cells. The biotransformation of the structural configuration of the cardiac glycoside compound bufadienolides isolated from *Cleome rutidosperma* possesses to have cytotoxic activity against the breast cancer cells. The compound bufotalin isolated from the *Cleome rutidosperma* methanol mediated extract was bio-transformed by the metabolites of the plant. The structural elucidation reveals the bio-transformed compound with potential cytotoxic activity. The isolated compound obtained was studied against the MCF-7 breast cancer cells for their cytotoxic nature. Bufotalin isolated from the leaf extracts shows potential cytotoxic activity against tested cells with an IC_{50} value of 0.0039 µmol/ml, it is essential to evaluate the potential of the compound in vivo.

CONCLUSION

The leaf methanol extract of *Cleome rutidosperma* and the isolated compound Bufotalin has potential cytotoxic activity against MCF-7 breast cancer cells. The activity of the isolated compound is much higher when compared with the crude extract of the tested plant. The inhibition of cancer cell proliferation and loss of cell viability is varying with the time and dose dependent manner with crude and isolated compound. Further studies may throw light

on the clinical application of plant derived bufotalin and the mechanism behind the biological action against the cancer cells.

Figures:

Figure: 1 TLC profiling of isolated compound from methanol leaf extract of *Cleome rutidosperma*

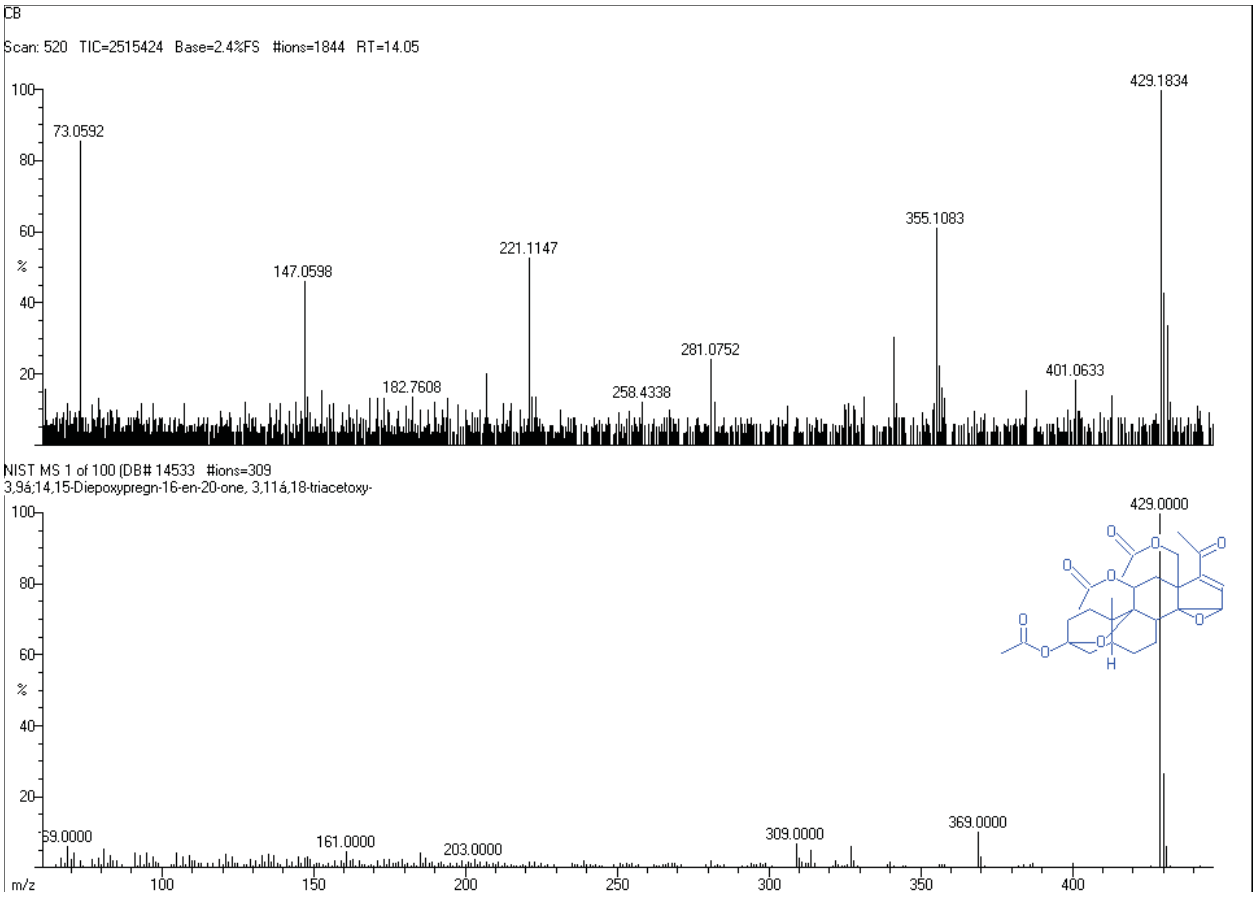


Figure: 2 Mass spectrogram of compound isolated from methanol leaf extract of *Cleome rutidosperma*

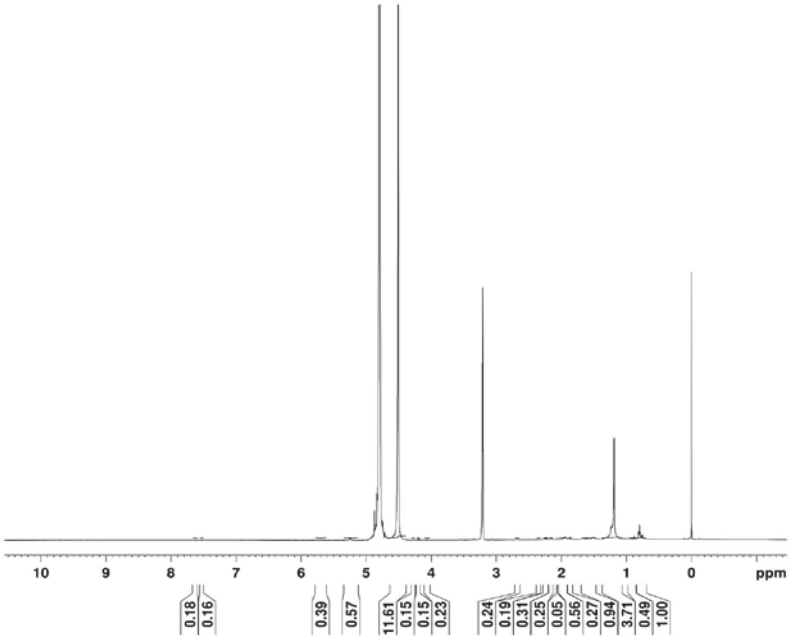


Fig. 3:[1]H NMR spectrum at 500 MHz in MeOD

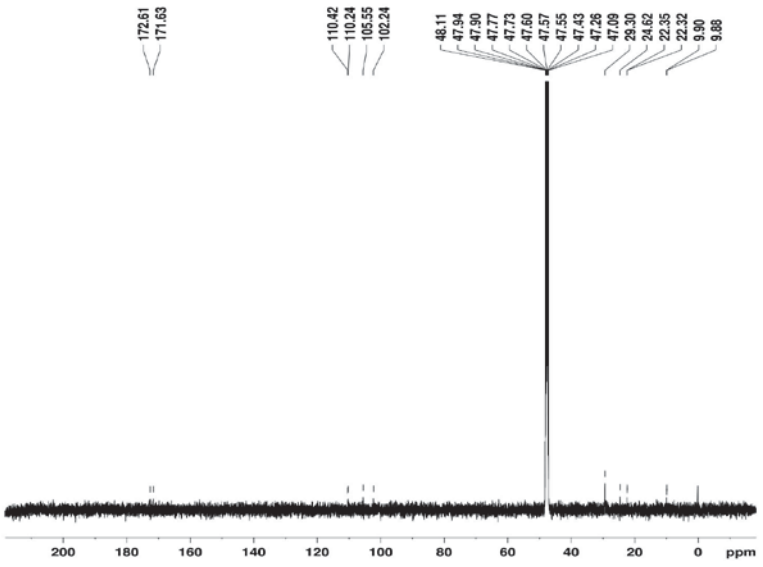


Fig. 4: [13] C NMR spectrum at 125 MHz in MeOD

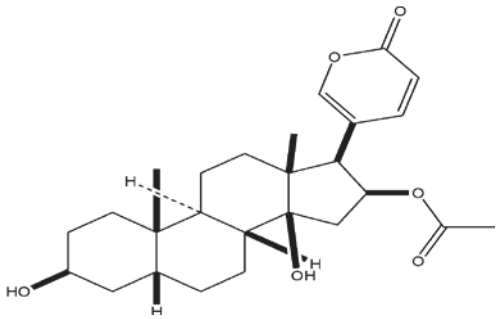
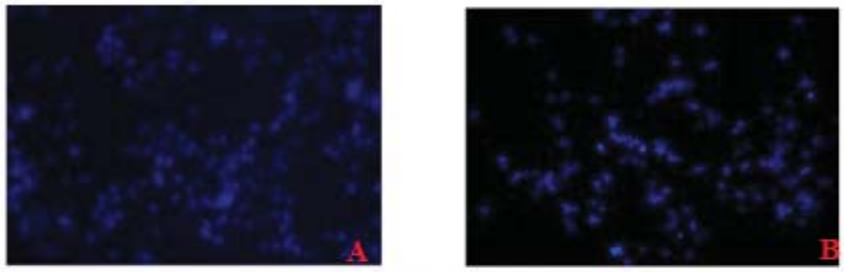
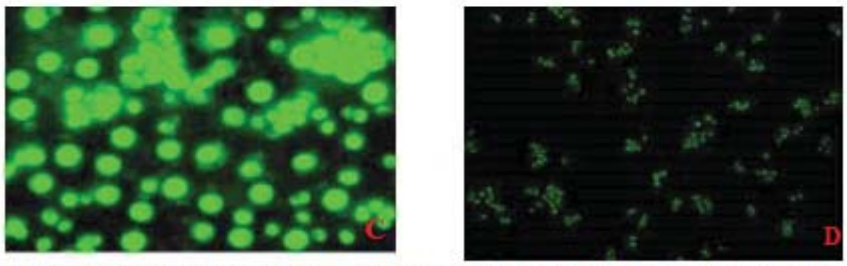


Fig. 5: Chemical structure of compound



Nuclear staining Using DAPI of breast cancer celllines (MCF-7) (a) untreated, (b) MCF-7 cells treated with Bufotalin isolated from Cleome rutidosperma methanol leaf extract



Staining with Acridine orange of Human Breast Cancer cells MCF-7 (c) Untreated (d) MCF-7 cells treated with Bufotalin isolated from Cleome rutidosperma methanol leaf extract

Figure 6: Invitro studies on the cytotoxic activity of Bufotalin isolated from the *Cleome rutidosperma* leaf extract.

Tables:

Table 1: FT-IR spectrum data of isolated compound

Functional group	Type of vibration	Region (cm^{-1})
O-H	O-H str	3418
C=C	C=C str	2053
C=O	α, β-Unsaturated carbonyl group (Six membered) / aliphatic C=O str	1637
C-H	C-H def	1384
C-H	C-H def	1323
C-O / C-O-H	C-O str / C-O str (3º alcohol)	1145
C=C-O-C / C-O-H	C-O str / C-O str (2° alcohol)	1101
C-H bend	Mono substituted alkene / Methylene rocking	621

Table 2: ^{1}H- and ^{13}C- NMR data of Bufadienolide

Bufadienolide (Bufotalin)					
Position No.	**^{1}H NMR (ppm)**	**^{13}CNMR (ppm)**	**Position No.**	**^{1}H NMR (ppm)**	**^{13}CNMR (ppm)**
Tetracyclic Hydrocarbon ring			Tetracyclic Hydrocarbon ring		
1	1.21 (t, 2H)	47.26	16	5.24 (q, 1H)	47.90
2	2.15 (q, 2H)	47.09	17	2.68 (d, 1H)	47.77
3	4.26 (m, 1H)	47.94	18	0.80 (s, 3H)	9.90
4	2.06 (t, 2H)	47.55	19	0.80 (s, 3H	9.88
5	2.05 (m, 1H)	47.73	3- & 14-(OH)	4.51(broad, s)	---
6	1.61 (q, 2H)	29.30	Pyran ring		
7	1.61 (q, 2H)	24.62	1	---	---
8	1.97 (q, 1H)	47.73	2	7.53 (s, 1H)	105.55
9	1.51 (t, 1H)	47.77	3	---	110.24
10	---	47.57	4	7.62 (d, 1H)	110.42
11	1.51 (q, 2H)	22.35	5	5.69 (d, 1H)	102.24
12	1.21 (t, 2H)	47.43	6(C=O)	---	171.63
13	---	47.74	Acetyl group		
14	---	48.11	1(C=O)	---	172.61
15	2.23 (d, 2H)	47.60	2	2.35 (s, 3H)	22.32

ppm-parts per million, ^{1}H-one proton,^{13}C-carbon thirteen

Table 3: Cytotoxicity effects of isolated compound of *Cleome rutidosperma* against MCF-7 breast cancer cell line by MTT assay

Concentration (µg/ml)	24hrs treatment		48hrs treatment	
	Cell Viability (%)	Cell Death (%)	Cell Viability (%)	Cell Death (%)
2	87.29±0.883	12.71	59.57±1.595	40.43
4	84.31±1.365	15.69	49.47±1.139	50.53
6	77.80±0.831	22.20	40.65±1.826	59.35
8	68.15±1.403	31.85	33.42±1.758	66.58
10	61.06±1.318	38.94	30.35±1.125	69.65
Control	100±0.00	0.00	100±0.00	0.00
IC_{50}	12.840		3.895	

Table 4: Cytotoxicity effects of methanol leaf extract of *Cleome rutidosperma* against MCF-7 breast cancer cell line by MTT assay

Concentration (µg/ml)	24hrs treatment		48hrs treatment	
	Cell Viability (%)	Cell Death (%)	Cell Viability (%)	Cell Death (%)
25	65.98±0.510	34.02	52.32±0.708	54.68
50	62.30±0.380	37.70	46.46±0.653	59.54
75	57.54±0.490	42.46	40.28±0.596	63.72
100	52.02±0.580	47.98	33.97±0.848	66.03
125	45.91±0.470	54.09	28.37±0.284	71.63
Control	100±0.00	0.00	100±0.00	0.00
IC_{50}	108.260		22.860	

ABBREVIATIONS

FTIR: **F**ourier Transform Infrared Spectroscopy
GC-MS: Gas Chromatography-Mass Spectrometry
NMR: Nuclear Magnetic Resonance
TLC: Thin Layer Chromatography
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
DMEM: Dulbecco's modified Eagle medium
FBS: Fetal bovine serum

DMSO: Dimethyl sulfoxide

NIST: National Institute of Standards and Technology

DAPI: 4-6-Diamidino 2-phenylindole

PBS: Phosphate Buffer Saline

SPSS: Statistical Package for Social Sciences

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