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HPLC Purification of bio-active *Cleome viscosa* and It's Anticancer Activity against Breast Cancer Cell Lines

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ABSTRACT

Cleome viscosa, often known as *Jakhya*, is a common weed that may be found in farms, gardens, and a variety of other areas. Because of its significant anti-disease effect, the plant and its parts have been employed in traditional medicine. Current study aim to purify the bioactive having potent antioxidant and anticancer activity against BT-474 cell lines. *Cleome viscosa* leaf methanolic extracts were purified through preparative HPLC and the isolated compounds were checked against MCF10A (normal cell line) and BT-474 (breast cancer cell line). HPLC isolated components from methanolic leaves extract included atropine, nevirapine, gallic acid, caffeic acid, vanillic acid, and kaempferitrin. Compounds were more efficient in terms of DPPH. Kaempferitrin inhibited DPPH by 94%. atropine, vanillic acid, and kaempferitrin were shown to be cytotoxic to BT474 cell lines, with IC_{50} values of 18.87, 1.316 and 46.42 µg/mL, respectively. Incubating with their IC_{50} concentrations caused no morphological harm to the cell. Only the number of cells decreased significantly as incubation time increased. *Cleome viscosa* purified compounds showed good antioxidant and anticancer activity. The compounds were found to be effective against BT474 breast cancer cell line.

Keywords: Anticancer activity, Bio-actives, Cell cytotoxicity, Cell viability, *Cleome viscosa*, HPLC purification.

INTRODUCTION

Cancer is one of the most vital causes of death worldwide[1,2]. Its frequency and death rate show its pace of rise in both emerging and developed nations[3,4]. Cancer incidence is steadily increasing, with 7.6 million deaths in 2008, and it is expected to quadruple by 2030[2]. Despite enormous expenditure and breakthroughs in cancer treatment, patient survival in many countries has not increased considerably[5]. There is no anticancer treatment that is 100% effective and has no negative effects, according to various publications[2-4]. As a result, there is still a critical need for novel drug development that results in competent anticancer medicines to combat the difficulties associated with chemotherapeutics, such as drug resistance and toxicity[3]. Because of the severe side effects of conventional chemotherapeutics,

great emphasis is being placed on plants as natural sources to improve cancer therapy[6,7]. More than 60% of licenced anticancer medications are natural products or derivatives of natural products; hence, herbs represent a substantial source of anticancer agents[8-11]. Over 3,000 plant species having anticancer activities have been identified as of today[12]. Plant materials are widely utilised across the world because they are more culturally acceptable, have less side effects, and are compatible with the human body[13]. Medicinal plants have traditionally been used to cure a variety of human ailments. The presence of phytochemicals, also known as plant metabolites, is generally the reason for this application[14]. According to several studies, plant anticancer activity is linked to a variety of natural chemicals such as catechins, polyphenols, and flavonoids.

Cleome viscosa is an ethnomedicinal important plant that have high traditional nutrient values. The plant is reported to have several bioactive compounds that can work against number of diseases. Several chemicals have been extracted from various parts of *Cleome viscosa*. *Cleome viscose* seeds are high in nutrients and include 26% oil, primarily Linoleic acid, palmitic acid, stearic acid, oleic acid, and linolenic acid, sugar, and seven important amino acids[15,16]. Methanolic extracts of *Cleome viscose* leaves has shown excellent antioxidant activity. The extract is shown to scavenge 43.33% of DPPH free radicals at minute concentration of 0.1 mg/mL[17]. Several studies on the antioxidant activity of *Cleome viscose* leaf extract have proved its therapeutic value as an antioxidant in a variety of ailments. Many molecules, including quercetin-3-O-β-D-glucopyranoside 7-O-α-L-rhamnopyranoside[18], cleomeside A, cleomeside B, cleomeside C and quercetin-3-O-[β-D-glucopyranosyl-(1-2)]-α-L-rhamnopyranoside 7-O-α-L-rhamnopyranoside exhibit antioxidant and anticancer properties[19]. Nevirapine is said to have anti-proliferative properties[20]. Lactam nonaic acid, which is present in roots, has antibacterial and cytotoxic properties[21,22]. Cleomaldic acid, present in seed and leaves, is said to be cytotoxic[23].

The present study is to determine the antioxidant and anticancer activity of bioactive purified from methanolic *Cleome viscosa* leaf extract against BT474 breast cancer cell and MCF10A as a normal cell line.

MATERIAL AND METHODS

MCF10 and BT-474 cell lines were purchased from NCCS, Pune. Leaves of *Cleome viscosa* were collected from Khandwa region of Madhya Pradesh, India and was authenticated by Dr. Anamika singh, Bardhman college, Bijnor. Leaves were washed, surface sterilized with 0.1% $HgCl_2$ and dried under shade for 2-3 weeks. Dried leaves were grounded to fine powder. Powder was then Soxhlet extracted following 4-5 cycle in methanol. Extract was evaporated to dryness and weighed. The dried extract was diluted in methanol to get a concentration of 500 mg/mL.

Crude extract was screened for their phytochemical constituents qualitatively and quantitatively. Phytochemical like alkaloids, flavonoids, phenolics and tannins were screened following the previous described methods[24]. While quantitative estimation of phenolic and flavonoid content was performed according to method of Singleton *et al.,* 1999[25] and Pontis *et al.,* 2014[26] respectively.

Thin layer Chromatography (TLC) of Leaf extract

Thin layer chromatography of extract was performed to evaluate number of phenolics and flavonoid. TLC plates were prepared by weighing ~3 g of silica gel G and mixing it in 7 to 10 mL of water to make slurry. The slurry was poured over cleaned 7.5*2.5 cm glass slides to make a thin layer of thickness not more than 0.25mm. The prepared plates were dried at room temperature followed by heating at 90-100^{0}C to activate silica in hot air oven. TLC chamber was saturated by solvent system[27] for 15 minute. Silica plates were spotted with 5-10 µL of extract. Dried the spot. The solvent system used for detection of flavonoid and phenolic groups are as followed. Solvent system for phenolic: Toluene: Acetone: Formic acid (4.5:4.5:1), Solvent system for flavonoids: n-butanol: Toluene: Ethyl acetate: Glacial Acetic acid (30:40:5). Run and dried the plate. Resolution of extract components was studied by locating different spots on a chromatogram using Folin-Ciocalteu reagent and sodium carbonate solution for phenolic content; and a mixture of 1% $FeCl_3$ and 1% potassium ferric cyanide for flavonoids. The distance between each spot and its application site was measured and recorded, and the R_f value was determined[28,29].

Purification of Bioactive compounds

Methanolic extracts were then purified, and the fractions were pre-screened for their anticancer activity.

Column purification of methanolic extract

Crude extract was dried and proceeded for column purification. Column was wet packed using silica gel 60-120 mesh. Silica gel slurry was prepared in hexane and poured over column having hexane solvent. Crude extract dried powdered was mixed with silica gel and poured slowly over the layer of packed silica avoiding disturbance of its layer. Increasing polarity was solvent was used to separated compounds in the basis of their polarity. The solvent used for the column purification was according to Bajpai *et al.,* 2016[30]. With some modifications. Hexane: 20 mL, Hexane: Chloroform (10:1): 22 mL, Hexane: Chloroform (5:1): 18 mL, Hexane: Chloroform (1:1): 20 mL, Hexane: Chloroform (1:5): 18 mL, Hexane: Chloroform (1:10): 22 mL, Chloroform: 20 mL, Chloroform: Methanol (10:1): 22 mL, Chloroform: Methanol (5:1): 18 mL, Chloroform: Methanol (1:1): 20 mL, Chloroform: Methanol (1:5): 18 mL, Chloroform: Methanol (1:10): 22 mL, Methanol: 20 mL. Fractions were collected at a constant flow rate. Total of 13 fractions were collected. Each of the fractions were screened for the phenolic, flavonoids anticancer activity. The fraction having high phenolic or flavonoid content were proceeded for HPLC purification.

Screening of crude and fraction for their anticancer activity

Anticancer activity of crude and column fractions was estimated by following the protocol of Mossman, 1983[31].

Isolation of single bioactive compound by preparative HPLC

Standards of flavonoids, phenolics and alkaloids (Atropine, Quercetin, Caffeic acid, Gallic acid, Kaempferitrin, Rutin, Nevirapine and Vanillic acid) were prepared at a concentration of 20 µg/mL. Fraction having significant % cytotoxicity against BT-474 was selected for preparative HPLC purification. Bioactive compounds were separated by preparative HPLC according to the protocol of Mansour *et al.,* 2017[32]. All the fractions and standards were filtered through 0.45µm PVDF syringe filters and sonicated at room temperature for 10 minute. Preparative HPLC was run on Agilent 1200 series equipped with dual pump and dual wavelength detector. Flow rate was maintained at 18 mL/minute. C18 reverse phase column (5µm, 20mm diameter, 250mm length) was used. Gradient solvent system for separation was used. The solvent system was as follow: Solvent A (H_2O/0.1% TFA), solvent B (Acetonitrile/0.1%TFA), Solvent A/Solvent B: 99: 1 (0-4 min), Solvent A/Solvent B: 99: 1 (4-12.8 min), Solvent A/Solvent B: 0: 99 (12.8-13 min), Solvent A/Solvent B: 10: 100 (13-55 min), Solvent A/Solvent B: 0: 100 (55-59 minute).

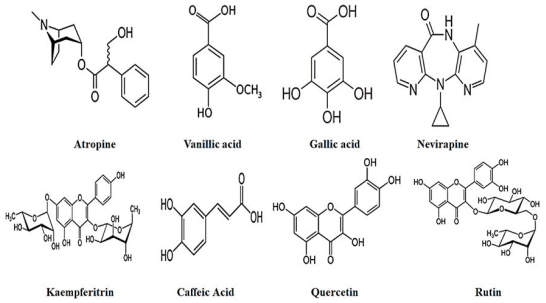

Fig. 1. Structures of selected alkaloids, flavonoids and phenolic compounds

Antioxidative assay of purified compounds

The purified compounds were assayed for their antioxidant activity against DPPH and ABTS free radicals. DPPH assay and ABTS assay was performed according to the protocol of Blois, 1998[33] and Pellegrini *et al.,* 1958[34] respectively. Briefly all the compounds were diluted to 1 mg/mL concentration and a positive standard of ascorbic acid of same concentration was also prepared for the antioxidant assay.

Cell cytotoxicity and cell proliferation assay of isolated components

HPLC purified samples diluted to 1 mg/mL concentrations were than tested for their cytotoxicity and cell proliferation assay. Cell cytotoxicity was estimated by following the protocol of Mossmann, 1983[31]. Cell proliferation was estimated by trypan dye exclusion assay[35].

Cell cytotoxicity assay

Cells were sub-cultured in 20 mL DMEM media with 10% FBS and incubated in CO_2 incubator with 5% CO_2 and 95% humidity at temperature of 35^{0}C. Cell were trypsinized after 70-80% confluency and proceeded for MTT assay.

2,5-diphenyl-2H-tetrazolium bromide (MTT) assay

Cytotoxicity of purified compounds were estimated by 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay following the protocol of Mossmann, 1983. The test samples were diluted in different concentrations of 100, 200, 300, 400, 500, 600 µg/mL concentrations. The diluted samples were mixed together with the cell lines that had been grown for 24 h in a confluent monolayer plate. After 72 h of incubation at 37°C in a 5% CO_2 incubator, the supernatant was collected, and 25 µL of MTT reagent (2 mg/mL) was applied to each well. Following a 2-h incubation period at 37°C, each well received 125 µL of dimethyl sulphoxide to solubilize the formazan precipitate, and the wells were agitated for another 15 minutes. At a wavelength of 490nm, the absorbance was measured using an ELISA reader. The control wells were medium only, DMSO control and Cell control (Well having BT-474 cells) without the examined chemical.

$\%Cell\ cytotoxicity=(A_0-A_1)*100$

Where: A_0 is the absorbance of the Cell (BT-474) control and A_1 is the absorbance of the well treated with diluted extracts.

Cell morphology and cell viability (trypan blue dye exclusion assay)

BT474 cell lines were incubated at the IC_{50} concentrations of the compounds. Six well microplates were seeded with ~3.4*105 cells/mL. Incubated for 24 hours. in CO_2 incubator with 5% CO_2, 95% air and 99% humidity at 37°C. Incubated with the IC_{50} concentrations of the compounds for 24, 48 and 72 hours. After each set of time of incubation, each of wells were examined under inverted microscope for their morphological differences. One control without any compound was also set. Cells were trypsin-zed and centrifuged for pelleting. Pellets were dissolved in 1 mL of DMEM media. Briefly 100-200 µL of the cells were mixed with equal volume of trypan blue (0.4%) and placed on haematocytometer. Observed under the compound microscope in 10X or 40X and counted the coloured (dead) and non-coloured (viable) cells. Counted all the four corners chamber of haematocytometer leaving the middle and below line and calculated the number of cells/ml using the formula:

Viable count (live cells/ml) = (Number of live cells/ numbers of total cells)* dilution*10000

RESULT

Methanolic extract of *Cleome viscosa* leaves were dark green colored and clear. Phytochemical screening of crude extracts showed presence of phenols, flavonoids, alkaloids and tannins (Table 1). These phytoconstituents act as defence molecule against biotic or abiotic stresses and are categorized into secondary metabolites[36].

Table 1: Phytochemical screening of major phytoconstituents

Phtochemical test		Result
Alkaloid	Mayer's test	++
	Dragendorff's test	-
	Wagner test	+
Flavonoid		++
Phenol		+
Tannins		+

++, +, - means strongly present, present, and absent respectively

Total phenolics was estimated to be 0.046 mg/g (Table 2) which is less than the results of Gupta *et al.,* 2011[37] and much greater than the findings of Govindan *et.al.,*2018[38]. Different phenolic content is due to different solvent used for extraction. Total flavonoids content was 0.042 mg/g which is greater than the previous findings[37,38].

Table 2: Total phenolic and total flavonoid content of crudes

Test	mg/g
Total phenolic content (mg GAE/g)	46±1.56
Total Flavonoid content (mg rutin/g)	42.5±3.05
Data are represented as mean± Std. dev.	

TLC analysis of *Cleome viscosa* crude for phenolics and flavonoids

Three flavonoids with R_f value of 0.86, 0.47 and 0.25 were found in the *Cleome viscosa* leaf extract. While number of phenolic compounds as observed by peaks numbers are also three with R_f values of 0.67, 0.49 and 0.124. TLC plate under long wavelength of UV indicates presence of seven fluorescent compounds with R_f values of 0.751, 0.612, 0.338, 0.299, 0.166, 0.114, 0.062. Their intensity indicating their concentrations can be observed by bar charts or chromatogram generated through JustTLC software (Figure 2).

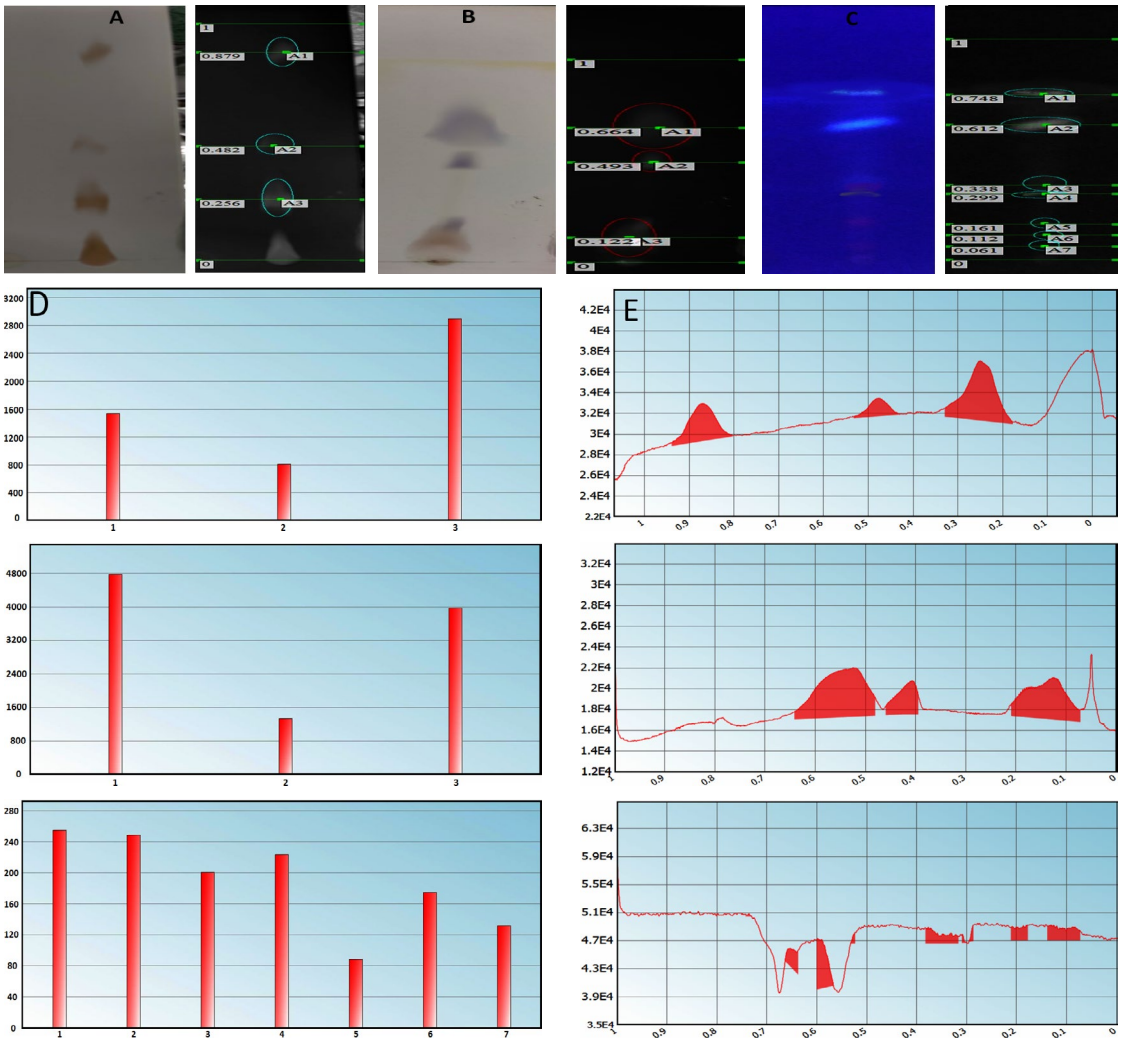

Fig. 2. Thin layer Chromatography of *Cleome viscosa*. A: Flavonoids, B: Phenolic, C: UV (Long wavelength), D: Graph between Volume (Y-axis) and number of spots in each plate, E: TLC profile of each plate (X-axis: Rf, Y-axis: Intensity)

Total of thirteen fraction were collected. Each of the fraction was proceeded for preliminary screening of phenolic and flavonoids along with their anti-cancer activity.

Table 3: Preliminary screening of fractions based on their phenol, flavonoid presence and %cytotoxicity in breast cancer cell line at a fix concentration of 1mg/mL

Fraction	Solvent (mL)	Phenols	Flavonoids	%Cytotoxicity at 1mg/mL coc. (BT-474)
F1	H (20)	-	+	7
F2	H: Chl (10:1)	-	+	8
F3	H: Chl (5:1)	-	-	10
F4	H: Chl (1:1)	-	-	21
F5	H: Chl (1:5)	+	-	20
F6	H: Chl (1:10)	+	-2	17
F7	Chl (20 ml)	+	-	27
F8	Chl: Meth (10:1)	+	+	18
F9	Chl: Meth (5:1)	+	+	14
F10	Chl: Meth (1:1)	++	+	38
F11	Chl: Meth (1:5)	+++	+	47
F12	Chl: Meth (1:10)	+++	+	49
F13	Meth (20)	+++	++	86

-, +, ++, +++ indicate absence, presence, moderate presence and strongly present respectively. H: Hexane, Chl: Chloroform, Meth: Methanol solvents

Out of thirteen fractions only last methanolic fraction having highest phenol and flavonoid presence and maximum percentage cytotoxicity was chosen for preparative HPLC purification of single compound (Table 3).

Preparative HPLC purification of selected fraction

The selected methanolic fraction was further HPLC purified to isolate single components.

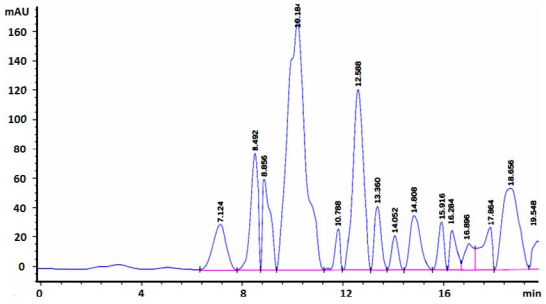

Fig. 3. HPLC profile of F13 fraction

HPLC profiling of the F13 fraction indicated the presence of 14 compounds. The compounds were identified by comparing the retention time of each peak with that of standards (Table 4). The identified compounds are tabulated in Table 5. Several bioactive were identified through HPLC profiling of *Cleome viscosa* leaf extract, including gallic acid, quercetin, catechin, chlorogenic acid, p-hydroxybenzoic acid, coumaric acid, viscosin, kaempferitrin, cleomeolide, nevirapine, cleomiscosin A and B, and several others.[39,40]

Table 4: Standard phenolic, flavonoid and alkaloids with their retention time, area of peak and response factor

Secondary metabolites	R. T.(min)	Area(mAU)	Response factor
Atropine	7.124	156	7.8
Nevirapine	18.656	62.2	3.11
Caffeic acid	14.852	1112.01	55.6
Gallic acid	16.092	1654.02	82.7
Quercetin	7.092	204.01	10.2
Kaempferitrin	12.588	79.02	3.95
Rutin	13.728	1920.02	96
Vanillic acid	9.968	659.02	32.95

Table 5: Identified secondary metabolites with their retention time, area of peak and concentration

Compound	R. T.(min)	Area(mAU)	Conc. (µg/mL)
Atropine	7.124	589.21	75.54
Unidentified	8.492	1020.02	
Vanillic Acid	10.184	2561.24	77.73
Unidentified	10.788	394.551	
Unidentified	14.052	174.942	
Gallic Acid	15.916	222.033	2.68
Nevirapine	18.656	790.569	254.2
Kaempferitrin	12.588	991.254	250.87
Caffeic Acid	14.808	413.563	7.44
Unidentified	16.284	193.613	

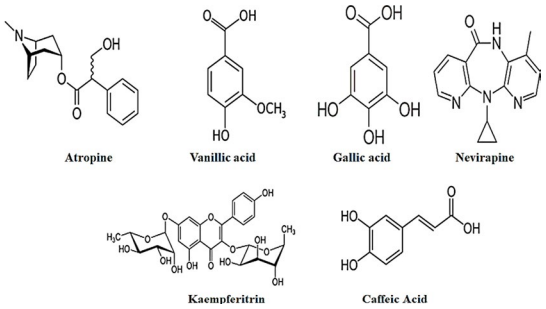

Fig. 4. Structures of isolated major compounds of *Cleome viscosa* leaf fraction

Antioxidative activity of purified compounds

DPPH and ABTS scavenging activity of all the purified (known and unknown) were estimated. Rather than ABTS, all the isolated compounds were shown to be more efficient against the DPPH free radical. Kaempferitrin was found to have maximum DPPH scavenging activity with 94% DPPH inhibition. While nevirapine was found to inhibit 76% of ABTS free radicals (Fig. 5). The unidentified compounds were not showing effective antioxidant activity.

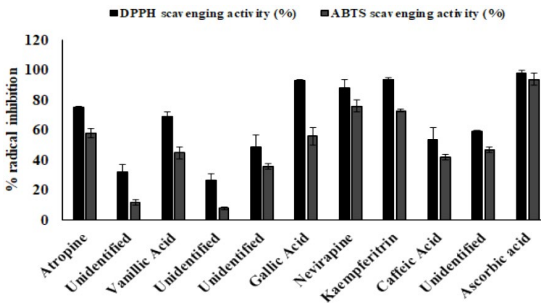

Fig. 5. Antioxidant activity of the purified compounds. Data are in mean ± SD. Where n=3

Anticancer activity and cell cytotoxicity of the purified compounds

All the compounds were tested against BT-474 cell lines for their potent anticancer activity. Fifty percent inhibitory concentration of all the compounds were calculated. Out of six selected compounds based on their antioxidant activity namely, Atropine, Vanillic acid, Gallic acid, Nevirapine, Kaempferitrin and Caffeic acid only three showed potent anticancer activity and exhibited good cell cytotoxicity against BT474 cell lines. These compounds are Atropine, Vanillic acid and Kaempferitrin with the IC_{50} values (concentration of compounds that results in 50% cell viability for MCF10A and BT474 cells) values of 18.87, 1.316 and 46.42 µg/mL respectively (Figure 6).

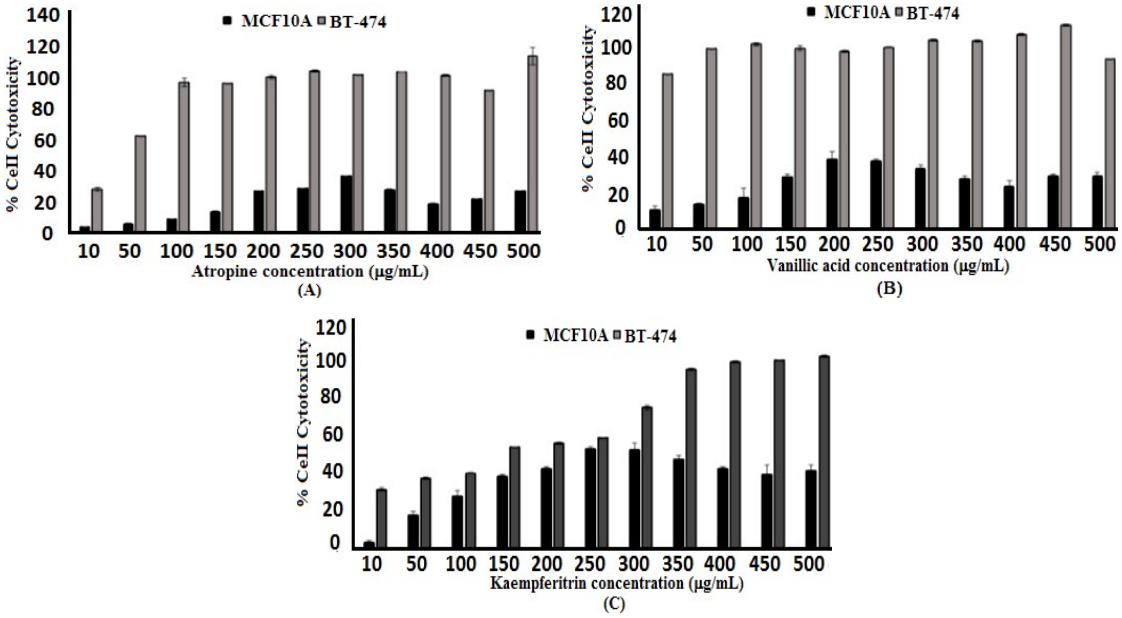

Fig. 6. Cell cytotoxicity assay of A: Atropine, B: Vanillic acid and C: Kaempferitrin. All the data are in mean ± SD. Where n=3

Cell morphology and cell viability at IC_{50} concentration of purified compounds

The BT474 cells treated with the IC_{50} doses revealed a substantial drop in cell number after 0, 24, 48, and 72 h of incubation (Fig. 7). There was no discernible morphological change between the treated and untreated cell. Cell viability falls dramatically as incubation time increases, with the exception of cells treated with the IC_{50} concentration of atropine between 24 and 48 h of incubation, where there is no significant loss in viability with time.

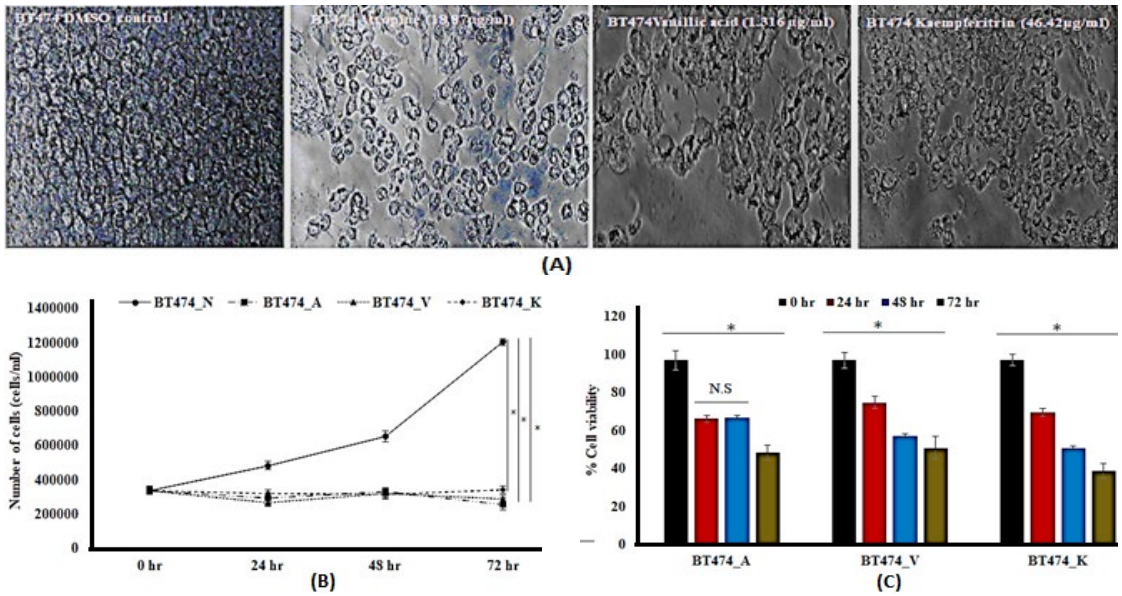

Fig. 7. A: Cell morphology after treatment of IC_{50} concentration of purified compound after 72 h of incubation. B: Graph showing cell number (cell/ml) after treatment with compounds at different time of incubations. C: Cell viability percentage after treatment with compounds at different time of incubations. Data are mean±SD. Where n=3,* indicate significant difference between and within the groups and N.S indicate no significant difference between the sample

CONCLUSION

Cleome viscosa is an ethnomedicinal significant plant with a large number of potent bioactives that can help with a variety of diseases. Atropine, vanillic acid, gallic acid, nevirapine, kaempferitrin, caffeic acid, along with some unidentified compounds, were purified from the methanolic extract of *Cleome viscosa* leaves. Kaempferitrin was found to have maximum antioxidant activity. All of the purified compounds having significant antioxidant activity also have potential anticancer activity against breast cancer cell lines, but three compounds, namely Atropine, Vanillic acid, and Kaempferitrin, have maximum cell cytotoxicity against the breast cancer cell line. From the results, it was concluded that isolated Kaempferitrin has good antioxidant and anticancer activity. Although Atropine has a high anticancer activity, it has a low antioxidant capacity.

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Conflict of interest

The authors state that they have no financial or other conflicts of interest.

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