Isolation of Cell cycle delay, Pro-metaphase arrest, and c-metaphase inducing agents from leaf aqueous extract of *Clerodendrum viscosum* Vent.

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Abstract:

Clerodendrum viscosum is a traditionally used medicinal plant and the earlier reports indicate its leaf aqueous extract (LAECV) contains metaphase arresting, cell cycle delay, and mitotic abnormality inducing active principles. The present study aimed to isolate metaphase arresting, cell cycle delay and c-metaphase inducing active principles of LAECV. The LAECV was fractionated with petroleum ether, chloroform and ethyl acetate and the respective extract fractions - AQPEF, AQCHF, and AQEAF were tested for *Allium cepa* root growth retardation; *A. cepa* and *Triticum aestivum* root swelling patterns. Based on root growth retardation activity, the AQPEF was selected and tested for its metaphase arresting, cell cycle delay, and c-metaphase inducing activities on *A. cepa* root tip cells and its phytoconstituents were analyzed through HPLC, LC-MS, GC-MS, and IR. The extracts induced root growth retardation and swelling patterns indicate the bioactive principles are effectively fractionated with petroleum ether solvent. The AQPEF induced colchicine like metaphase arrest, cell cycle delay, and c-metaphase in *A. cepa* root tip cells and the GC-MS analysis detected the presence of clerodane diterpenoids like clerodin (m/z 434.3), 15-hydroxy-14, 15-dihydroclerodin (m/z 452), 15-methoxy-14, 15-dihydroclerodin (m/z 466), and 14, 15-dihydroclerodin (m/z 436) with the retention time of 14.038, 14.103, 14.480 and 14.655 respectively. Thus, the present study explores clerodane diterpenoids of LAECV as metaphase arresting, cell cycle delaying and c-metaphase inducing active principles.

Keywords: Diterpenoids; Mitotic abnormality; Allium cepa; Metaphase arrest.

1. Introduction:

Clerodendrum viscosum (Family: Lamiaceae) is widely distributed throughout Asia, Africa, Australia, and America. It is used in traditional, Ayurvedic, Homeopathic, and Unani medicine (Nandi and Lyndem 2015). The whole plant juice is used against worm infection, cough, itching, leprosy, scorpion sting, asthma, bronchitis, fever, *etc.* (Bhattacharya 1981, Kirtikar and Basu 1991). The plant is well known for its effectiveness against rheumatism in Unani medicine (Singh et al. 1997). It is prescribed to treat postnatal complications, diarrhea, and fresh wounds in the Indian Homeopathic system (Hamilton 1997, Nadkarni and Nadkarni 2002). The different parts of this plant are used as a remedy for asthma, malaria, cataract, diseases of the skin, blood, and lung by the Indian Tribals of Chotanagpur plateau (Kirtikar and Basu 1991). Its bark juice is used to relieve indigestion and abdominal pain. The plant's ethanolic extracts have antioxidant, antimicrobial, hepatoprotective, wound healing, and antidiarrheal activities (Bhattacharjee et al. 2011).

The anthelmintic activity was reported in leaf ethanolic, methanolic, and aqueous extracts against *Pheretima posthuma* (Islam et al. 2013). The leaves and roots have great potential against different microbial and fungal strains. Acetone and chloroform extracts of *C. viscosum* leaves have an inhibitory effect on the growth of *Shigella sp*., *Vibrio cholerae*, *Klebsiella pneumonia*, and *Pseudomonas aeruginosa*, *etc*. (Lobo et al. 2010) while ethanolic fraction has shown antifungal activity against *Aspergillus niger*, *A. flavus*, and *Candida albicans* (Modi et al. 2010). A saponin isolated from petroleum ether extract of leaves has analgesic activity (Sannigrahi et al. 2009). In vivo antinociceptive activity of methanolic extract is comparable to diclofenac sodium drug (Rahman et al. 1970). Reduction in CCl_4 induced hepatotoxicity on rats after treatment with methanolic leaf extract reveals its hepatoprotective activity which is further supported by biochemical blood parameters (Sannigrahi et al. 2009). Successive ethanolic extract of *C. infortunatum* shows highest antioxidant activity compared to petroleum ether and chloroform extracts, whereas significant wound healing activity was exhibited by petroleum ether and ethanol extract. These pharmacological effects were also correlated with total phenolic content of

the plant (Gouthamchandra et al. 2010). Leaf methanolic extract exerts a restoring effect on blood glucose levels after streptozotocin treatment (Arvind et al. 2002, Das et al. 2011). Allelochemicals from leaf aqueous extract have been found to harm the growth and germination of weeds in agro-ecosystem (Qasem and Foy 2001, Devi et al. 2013). A promising positive correlation is established between the plant's parts and their insect repellent and insecticidal activity (Muh et al. 2014).

Acute toxicity test reveals that these plant parts are safe up to 2000 mg/kg body weight (Gupta et al. 2012). The crude leaf extracts contain phenolics viz. fumaric acids, acetoside, methyl esters of caffeic acids, terpenoids like clerodin, flavonoids such as apigenin, acacetin, scutellarein, quercetin, hispidulin, steroids such as clerodone, clerodolone, clerodol, clerosterol and some fix oils containing linolenic acid, oleic acids, stearic acid and lignoceric acid (Singhmura 2016).

In our previous study, we have reported that treatment with leaf aqueous extract of *C. viscosum* (LAECV) on root apical meristem cells of wheat and onion gave an increased metaphase frequency along with a reduction in mitotic index, antiproliferative, and apoptosis-inducing effects (Ray et al. 2012). The metaphase arrest and cell cycle delay inducing effects were somewhat comparable to Colchicine's actions (Ray et al. 2013, Kundu and Ray 2016, Roy and Ray 2017).

Colchicine inhibits spindle formation in cells which leads to generating signals delaying the transition from metaphase to anaphase (Salmon et al. 1984). Later overtime when the concentration of colchicine decreases in the environment, the chromatids separate abnormally and the plant cells become polyploid (Caperta et al. 2006). Colchicine treatment to onion root apical meristem cells results in root growth inhibition, roots' swelling, haphazardly arranged condensed chromosomes and along with an increased frequency in metaphase (Ray et al. 2013). In another comparative study, colchicine and LAECV treatments on *A. cepa* root tip cells revealed their similar cytotoxic effects (Kundu and Ray 2016). The metaphase arrest and cell cycle delay inducing effects of LAECV raised the key question about its active principle(s) (Ray et al. 2013). Therefore, this study aims to isolate the bioactive molecule(s) of LAECV. The LAECV was successively fractionated with petroleum ether, chloroform, ethyl acetate, and all the extract fractions were tested for *Allium cepa* root growth inhibition and *A. cepa* and *T.*

aestivum root swelling patterns. The petroleum ether fraction (AQPEF) was selected for further analysis including cell cycle delay, pro-metaphase arrest, and c-metaphase inducing effects on *A. cepa* root tip cells and its bioactive principle(s) were detected.

2. Materials and methods

2.1 Chemicals

Orcein, methanol, and glacial acetic acid were obtained from Merck, Germany. Petroleum ether, Chloroform, and Ethyl acetate were obtained from Thermo Fisher Scientific, The USA. Other analytical grade chemicals were obtained from reputed manufacturers.

2.2 Plant collection, aqueous extraction and solvent fractionation

The fresh *C. viscosum* leaves were collected from Burdwan University campus, West Bengal, India, and it was taxonomically identified by Professor A. Mukherjee, Department of Botany, The University of Burdwan. A voucher specimen (No. BUTBSR011) is maintained in the Department of Zoology, B.U., for future reference.

Fresh leaves were washed in tap water, dried in shade, ground by Philips Mixer Grinder HL1605, and the obtained powder was stored in a tightly sealed container for further use. 100 g of this pulverized material was extracted in 2.5 L of boiling distilled water for 2-3 h and the extract was filtered with filter paper and the extract coded as leaf aqueous extract of *C. viscosum* (LAECV).

The LAECV was successively fractioned by petroleum ether (AQPEF), chloroform (AQCHF) and ethyl acetate (AQEAF) with the help of a magnetic stirrer for 10-12 h (Figure 1). After collection by siphoning, the respective solvent fractions were concentrated by rotary vacuum evaporator and stored in a glass container.

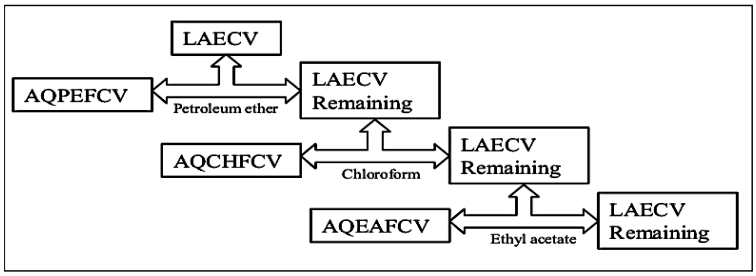


Figure 1: Schematic diagram of successive fractionation of LAECV with petroleum ether, chloroform and ethyl acetate.

2.3 Experimental plants

Allium cepa and *Triticum aestivum* root apical meristem were used as plant models for determining the root growth retardation and root swelling pattern for selecting the most effective extract fraction and then its cell cycle modulation and metaphase arresting activities were analyzed.

2.4 Root growth retardation and root swelling effects of the extract fractions

2.4.1 Culture and treatment of Allium cepa roots

1% sodium hypochlorite mediated surface-sterilized *A. cepa* bulbs were placed in 6-well plates containing distilled water and kept in the environmental test chamber for germination (25-27 °C, humidity 50%). The 48h aged similar-sized *A. cepa* roots (2-3 cm root length) were treated with 12.5, 25, 50, 100, and 150 µg/mL concentrations of AQPEF, AQCHF, and AQEAF continuously for 24, 48, and 72 h. Stock solutions of fractions were prepared in DMSO. The experiments were performed in triplicate. For root swelling pattern analysis, the 24 h aged onion roots were treated with the extract's fractions for 4 h and allowed to grow for another 16 h in water and root tip swelling patterns were analyzed.

2.4.2 Culture and treatment to wheat seedlings for root swelling pattern analysis

The similar-sized seeds of *T. aestivum* were surface sterilized with 1% sodium hypochlorite solution and five replicas of each with 30 seeds were prepared for each treatment. Seeds were placed on filter paper kept in sterilized Petri dishes (90 mm), were covered then incubated at 25°C in a dark culture room, for germination. For the determination of active fractionation, the LAECV (4 mg/mL) and its extract fractions were treated to 24 h aged wheat roots initially for 4 h and then allowed for another 16 h as recovery and the root swelling patterns were observed.

2.5 Cell cycle delay, pro-metaphase arresting and c-metaphase inducing effects of AQPEF

2.5.1 Allium cepa root sprouting.

For root sprouting, the similar size *A. cepa* bulbs were surface sterilized by 1% sodium hypochlorite and the bulbs were placed in 6-well plates containing distilled water and kept in dark at 25-27°C within an environmental test chamber.

2.5.2 Treatment and preparation of mitotic phases from root apical meristem cells

The 48h aged similar-sized *A. cepa* roots (2-3 cm) were treated with 50, 100, 150, 200 µg/mL of AQPEF and 150 µg/mL of colchicine for 2 and 4 h. Stock solution of Petroleum ether fraction was prepared in DMSO for treatment. After 2 and 4 h exposure, 8-10 roots were fixed and processed for squash preparation following the standard procedure (Chaudhuri and Ray 2015). The remaining roots were allowed to grow further for another 16 h in distilled water and subsequently, root tips were fixed. The control group, which had not received any treatment, maintained in distilled water simultaneously with the treatment groups. The treated and untreated root tips were fixed in aceto-methanol (3 parts methanol: 1part glacial acetic acid) for 24 h and then hydrolyzed for 10 min in 1 N HCl at 60˚C. Furthermore, roots were stained with 2% aceto-orcein and finally squashed in 45% acetic acid (Sharma and Sharma 1999, Ray et al. 2013). The well-spread areas of squashed roots were focused under the bright field light microscope for observation and scoring the cell division phase and c- metaphase frequencies.

2.6 Preliminary phytochemical detection of organic solvent extract fraction of LAECV

Phytochemicals like flavonoids, alkaloids, terpenoids, anthraquinones, tannins, saponins, steroids, phlobatannins, glycosides, and carbohydrate were analyzed in AQPEF, AQCHF and AQEAF by following the standard method of Harborne 1973, Trease and Evans 1989 and Sofowora 1993 (Harborne 1973, Trease and Evans 1989 and Sofowora 1993).

2.7 Characterization of AQPEF by UV-VIS spectrophotometer, FT-IR Spectroscopy, HPLC, LC-MS, and GC-MS

The AQPEF was dissolved in petroleum ether and UV-Vis spectrophotometric analysis was done by UV-Vis Spectrophotometer (Shimadzu Corporation). Analytical reverse-phase high-performance liquid chromatography (HPLC) was carried out with a 600 series pump and C-18 column (Hitachi). The separation was done in isocratic mode with HPLC-grade acetonitrile and water as a mobile phase at the ratio of 70:30, with a flow rate of 1mL/min. The samples were filtered through a 0.22 μm syringe filter (Himedia) and a 10 μL volume of sample was injected via the injector. The AQPEF was air-dried in a sterile Petri dish to get a yellowish powdery material which was then subjected to FT-IR spectroscopy by IR Prestige, Shimadzu. The Sample was dissolved in HPLC-grade methanol and filtered using a 0.22 μm syringe filter (Himedia). LC-MS was carried out using a mass spectrometer (AB-Sciex) and GC-MS was performed using a mass spectrophotometer of Agilent Technologies from TCG life science private limited, Kolkata, India.

2.8 Scoring and statistical analysis

Allium cepa root growth retardation effect of the different organic solvent fractions of LAECV was performed in triplicate and data analyzed with student t-test. In the case of squash preparation of *A. cepa* root apical meristem cells, at least three randomly coded slides were observed under the light microscope. Calculation of the mitotic index was done by counting the number of dividing cells per total cells scored for each concentration. Different cell phase frequencies, mitotic index, and c-metaphase cells were analyzed by 2X2 contingency $\chi 2$-test. Pearson multivariate correlation analyzed using GraphPad Prism 8.4.3.

3. Results

3.1 Allium cepa root growth retardation effects of extract fractions of LAECV

Data indicate that AQPEF, AQCHF, and AQEAF induced concentration-dependent *A. cepa* root growth retardation effects. The root growth and growth rate retardation effects were maximum in the case of AQPEF treated samples. In 72 h treated samples, the root length retardation percentages were 68.51±0.56, 60.02±0.94, and 51.20±0.50% respectively for AQPEF, AQCHF, and AQEAF at a concentration 150 μg/mL (TableS1). Similarly, the root growth rate retardation percentages were 91.01±1.51, 89.86±1.60, and 89.85±2.09 % respectively for AQPEF, AQCHF, and AQEAF. IC50 values for root growth rate retardation were 23.68±5.5, 62.78±3.26, and

106.15±4.03 µg/mL respectively for AQPEF, AQCHF, and AQEAF at 48 h of treatment (Figure 2).

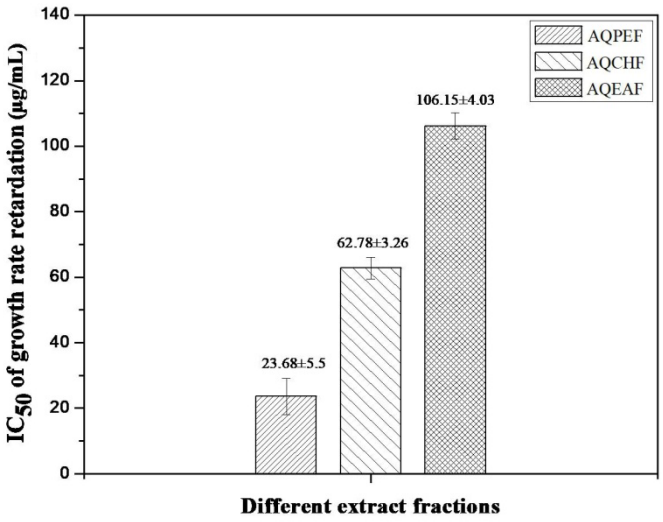


Figure 2: IC50 values (µg/mL) on onion root growth rate retardation of AQPEF (petroleum ether fraction), AQCHF (chloroform fraction), and AQEAF (ethyl acetate fraction). Data represented as mean ± sem. [a]significant at $p<0.001$ and [b]significant at $p<0.01$, [c]significant at $p<0.05$ as compared to their respective control by t-test with respective df = 1.

Therefore, in the second set of experiments where our previously standardized procedure wheat roots were exposed with LAECV and its extract fractions for 4 h and followed 16 h by a recovery phase, LAECV, AQPEF, and AQCHF showed a characteristic swelling pattern. Amongst the extract fractions, the petroleum ether fraction seemed to be the most active fraction in terms of root swelling (Figure S1).

3.2 AQPEF induced cell cycle delay in Allium cepa root tip cells

3.2.1 Mitotic index

Data indicate that the mitotic index percentage in root apical meristem cells varies with the growing periods and extract concentrations. In the present study, the highest MI (13.25±0.72%) scored at 4 h of AQPEF (150 µg/mL) treatment and the lowest MI (3.55±1.10%) of colchicine

(150 µg/mL) at 4 h followed by 16 h recovery samples. At 2 h, MI% were gradually increased- 5.67±0.30, 6.63±0.41, 7.58±0.5, 9.76±0.26, and 10.51±0.80% respectively for 0, 50,100, 150, and 200 µg/mL. The MI also increased in 4 h treated samples - 7.09±0.62, 10.3±0.56, 10.3±0.29, 13.25±0.72, and 9.95±0.80% respectively for 0, 50,100, 150, and 200 µg/mL of AQPEF. In the case of 16 h recovery samples, MI% were 10.06±0.92, 7.00±0.29, 8.45±1.64, 6.51±0.77, and 7.43±0.38% respectively for 0, 50, 100,150 and 200 µg/mL of AQPEF. In the case of 150 µg/mL colchicine treatment, data indicate the MI% decreasing tendencies at 2 h (12.55±1.44%), 4 h (9.17±0.56%) and 4 h followed by 16 h recovery samples (3.55±1.10%).

The maximum increase in MI % (13.25±0.72) was scored from the 150 µg/mL concentration of AQPEF treatment at 4 h. In contrast to these MI% increasing tendencies, a decreased percentage was observed in AQPEF treatment at 4 h followed by 16 h recovery samples. The lowest MI % was found in the case of 150 µg/mL (6.51±0.77%) treatment followed by 50 µg/mL (7.00±0.29 %) (Table 1).

3.2.2 Frequency of Prophase

Data indicates that AQPEF (50, 100, 150, and 200 µg/mL) treatment for 2 h could not significantly alter the frequency of prophase, though a decreasing tendency was observed. Statistically significant ($p<0.05$, 0.001) reduction in prophase frequency was observed in the case of 4 h of AQPEF treatment and 4 h treatment followed by 16 h recovery samples. The lowest prophase percentage (8.72±2.61) was observed in 50 µg/mL concentration of AQPEF treatment for 4 h. 150 µg/mL of colchicine treatment, significantly decreases the prophase frequencies at 2 h (11.91±1.41%) and 4 h (9.35±1.92%) compared to untreated, whereas marked recovery of prophase frequency (23.35±3.4%) can be seen in 4 h followed by 16 h recovery samples (Table 1).

3.2.3 Frequency of Metaphase

The AQPEF treatments (50, 100, 150, and 200 µg/mL) induce an increased frequency of metaphase at 2 and 4 h treatments, and 4 h treatment followed by 16 h recovery samples. The highest metaphase cells percentage (81.44±1.61) was scored from 4 h AQPEF treated (100 µg/mL) samples and that was followed by the concentration of 150 µg/mL (78.48±2.41%).

However, in the case of 16 h recovery samples, the metaphase frequency showed a reducing tendency for 4 h treated samples and the metaphase frequencies were scored as 65.69±3.87 % and 52.42±6.02 % respectively for the concentration of AQPEF 100 and 150 μg/mL. Colchicine treatment for 2 h and 4 h notably increases the metaphase frequency to 79.75±4.46% and 84.88±1.26 % respectively. In the case of 6 h recovery after 4 h colchicine treatment, the metaphase frequency (38.81±1.66) comparable to untreated control (Table 1).

3.2.4 Frequency of Anaphase

The percentage of anaphase cells decreased significantly ($p<0.0001$) at 4 h of AQPEF (50, 100, 150 & 200 μg/mL) treated samples. The lowest anaphase percentage (6.67±1.04%) was observed in 100 μg/mL concentration of AQPEF at 4 h. The decreased anaphase frequency was also observed in 2 h treated samples, though there was no significant difference as compared to the control group. In the case of 16 h recovery samples, the anaphase percentage reducing tendency was observed for the concentration of 100 (12.23±0.72%) and 150 μg/mL (18.64±2.49%), whereas the remaining concentrations, 50 and 200 μg/mL shown an increased anaphase percentage respectively 24.58±0.94% and 29.68±2.72%. Similarly, colchicine treatment (150 μg/mL) for 2 h and 4 h significantly reduced the anaphase frequency to 5.98±2.74% and 4.70±0.63% respectively and conversely in 16 h recovery samples, the anaphase frequency (11.60±2.05) increased (Table 1).

3.2.5 Frequency of Telophase

The AQPEF (50, 100, 150 & 200 μg/mL) treatment showed a decreased tendency of telophase frequency in each of the treatment hours. However, the significant difference ($p<0.0001$) was observed at 4 h treated samples, where the 0, 100, 150, and 200 μg/mL concentrations of AQPEF induced decreased telophase percentages 12.45±0.51, 1.83±0.52, 3.39±0.76, and 2.26±0.48 % respectively. Except in 150 μg/mL concentration, the reduced anaphase frequency was also observed at 2 h and 4 h followed by 16 h treated samples, though the differences in the frequencies were not significant as compared to control. Colchicine (150 μg/mL) treatment for 2 h and 4 h significantly reduced the telophase frequency to 2.32±0.49% and 1.00±0.07% respectively, whereas in 16 h recovery samples it increased (26.20±3.57) (Table 1).

3.2.6 C-metaphase frequency

The study result showed that the C-metaphase was found to be the most frequent type of abnormalities induced by AQPEF in *A. cepa* root apical meristem cells. The AQPEF treatment for 2 and 4 h induces a significant (p<0.001& 0.0001) increase in the frequency of C-metaphase in *A. cepa* root apical meristem cells. In the case of AQPEF treatments for both 2 and 4h, the C-metaphase frequency increased in a dose-dependent manner. The increased percentage of C-metaphase induced by 100, 150 & 200 µg/mL of AQPEF were scored respectively as 39.2±4.05, 55.62±2.03, and 45.15±3.86% at 2 h and 80.08±1.65, 70.86±2.74, and 68.88±1.67% at 4 h treated samples. In the case of 2 h treatment, 150 µg/mL concentration of AQPEF showed the highest C-metaphase frequency (55.62%) but in the case of 4 h treatment, 100 µg/mL concentration of AQPEF induced the highest (80.08%) C-metaphase. After 16h recovery treatment, a slightly decreased C-metaphase frequency was observed for those respective concentrations. Increase C-metaphase frequencies were also noticed in colchicine (150 µg/mL) treatment for 2 h and 4 h as 77.95±5.63% and 83.00±1.93% respectively. In 16 h recovery after colchicine treatment, c-metaphase frequency (9.96±3.40%) decreased (Table 1 & Figure 3).

Table 1: Effects of AQPEF (petroleum ether fraction) on the mitotic index and mitotic phase frequency in *A. cepa* root apical meristem cells

H	Conc(µg/mL)	Tc	Tdc	MI%	Pro%	Meta%	Ana%	Telo%	C-Meta%
2	0	1959	111	5.67±0.30	21.95±4.90	36.86±2.97	25.87±3.32	15.13±0.70	1.66±1.66
	50	2181	144	6.63±0.41	16.67±1.27	42.41±1.35	25.64±4.45	15.25±2.48	22.18±1.52^{a}
	100	2445	183	7.58±0.5 ^{c}	15.93±2.63	56.52±1.96	18.48±3.42	11.09±0.94	39.2±4.05 ^{a}
	150	2404	234	9.76±0.26 ^{a}	16.32±1.01	63.67±3.27^{c}	14.95±1.28	5.05±1.76^{b}	55.62±2.03^{a}
	200	2389	251	10.51±0.80^{a}	17.74±1.90	53.67±2.05	17.96±2.23	10.63±1.13	45.15±3.86^{a}
	150©	1987	248	12.55±1.44^{a}	11.91±1.41	79.75±4.46^{a}	5.98±2.74 ^{a}	2.32±0.49^{a}	77.95±5.63^{a}
4	0	2914	207	7.09±0.62	19.02±0.97	37.72±2.60	30.77±2.07	12.45±0.51	0.00±0.00
	50	3423	352	10.3±0.56 ^{a}	8.72±2.61^{b}	66.47±5.70^{a}	14.54±1.98^{a}	10.24±2.27	49.38±5.71^{a}
	100	2162	222	10.3±0.29 ^{a}	10.04±1.61^{c}	81.44±1.61^{a}	6.67±1.04 ^{a}	1.83±0.52^{a}	80.08±1.65^{a}
	150	3035	403	13.25±0.72^{a}	9.34±1.11^{c}	78.48±2.41^{a}	8.76±2.53 ^{a}	3.39±0.76^{a}	70.86±2.74^{a}
	200	3525	352	9.95±0.80 ^{a}	14.33±2.74	76.43±2.61^{a}	8.00±0.27 ^{a}	2.26±0.48^{a}	68.88±1.67^{a}
	150©	3286	301	9.17±0.56 ^{b}	9.35±1.92 ^{b}	84.88±1.26^{a}	4.70±0.63 ^{a}	1.00±0.07^{a}	83.00±1.93^{a}
4+16	0	2449	241	10.06±0.92	24.63±2.84	38.95±0.78	23.27±0.93	13.11±2.99	0.00±0.00
	50	3946	276	7.00±0.29 ^{a}	14.83±0.34^{c}	45.32±0.66	24.58±0.94	15.23±0.43	0.36±0.36 ^{a}
	100	2130	177	8.45±1.64	12.77±3.18^{c}	65.69±3.87^{b}	12.23±0.72^{c}	9.28±1.71	43.05±13.28^{a}

150	2068	136	6.51±0.77 [a]	20.08±0.92	52.42±6.02	18.64±2.49	8.82±3.99	33.73±1.91 [a]
200	2162	161	7.43±0.38 [b]	25.00±2.85	35.78±2.46	29.68±2.72	9.51±2.22	24.81±5.00 [b]
150©	1922	69	3.55±1.10 [a]	23.35±3.40	38.81±1.66	11.60±2.05	26.20±3.57	9.96±3.40 [a]

[a]Significant at $p<0.0001$ and [b] significant at $p<0.001$, [c] significant at $p<0.05$ as compared with their respective control by 2x2 Contingency $\chi 2$ -test with respective df = 1. H; Hours, Comp; Compound, Conc; Concentration, Tc; Total no of cells, Tdc; Total no of dividing cells, MI; Mitotic index, Pro; Prophase, Meta; Metaphase, Ana; Anaphase, Telo; Telophase, C-Meta; Colchicine like metaphase or C- metaphase, ©; Colchicine.

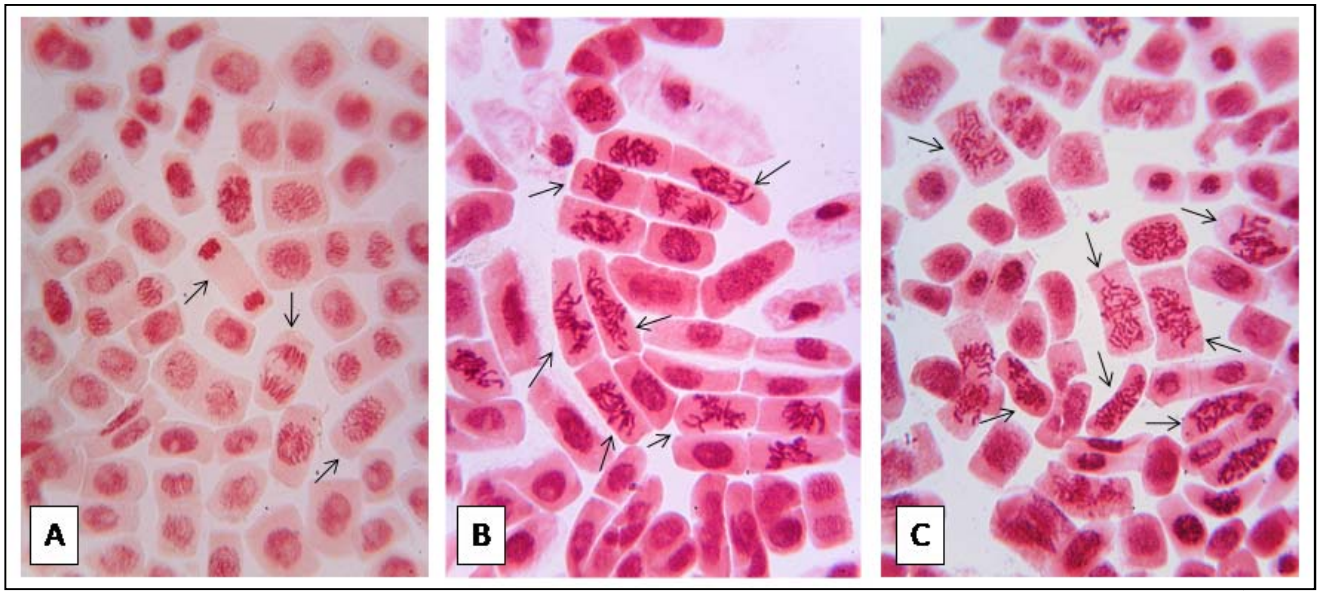


Figure 3: Photomicrograph showing the squash preparation of A, Untreated; B, AQPEF (petroleum ether fraction); C, Colchicine induced increased C-metaphase frequency in root apical cells of *A. cepa* at 4 h continuous treatment.

3.3 Correlation analysis

From Pearson multivariate correlation analysis in Colchicine (150µg/mL) and AQPEFCV (200µg/mL) among MI% (Mitotic Index%), PRO% (Prophase%), META% (Metaphase%), ANA% (Anaphase%), TELO% (Telophase%) and CM% (C-metaphase%) considering the change in their relative percentages at 2, 4 and 4+16 hour, a positive correlation was drawn between MI % and META % whereas a negative correlation was found between MI % and ANA % both in colchicine ($r = 0.89$ in META% and $r = -0.85$ in ANA%) and AQPEFCV ($r = 0.72$ in META% and $r = -0.80$ in ANA%). Negative correlation is found between MI% and each of

PRO% and TELO% in AQPEFCV which is similar to colchicine. Correlation between CM% and META% attains highest value (r = + 1.00) in both the cases (Figure 4).

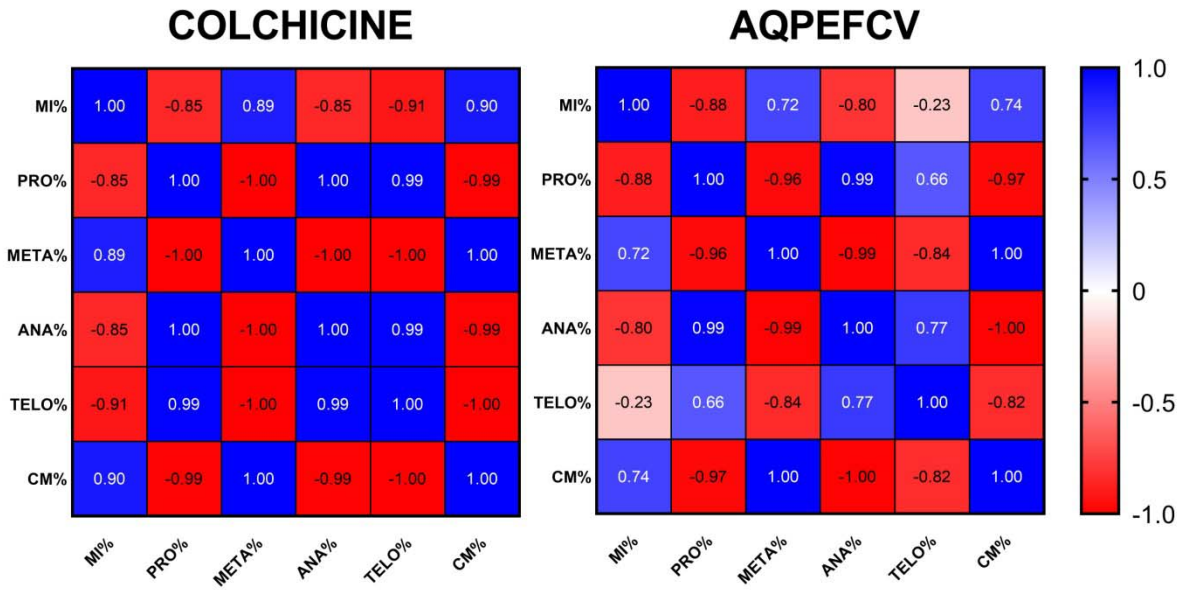


Figure 4: Pearson multivariate Correlation matrix in Colchicine (150µg/mL) and AQPEFCV (200µg/mL) among MI% (Mitotic Index%), PRO% (Prophase%), META% (Metaphase%), ANA% (Anaphase%), TELO% (Telophase%) and CM% (C-metaphase%) considering the change in their relative percentages at 2, 4 and 4+16 hour.

3.4. Phytochemical analysis

Preliminary phytochemical analysis indicates the presence of terpenoids in all fractions. Petroleum ether and chloroform fractions consist of saponins. Carbohydrates are present only in chloroform fraction. Alkaloids, flavonoids, steroids, tannins, glycosides are absent in all organic solvents fraction (Table 2).

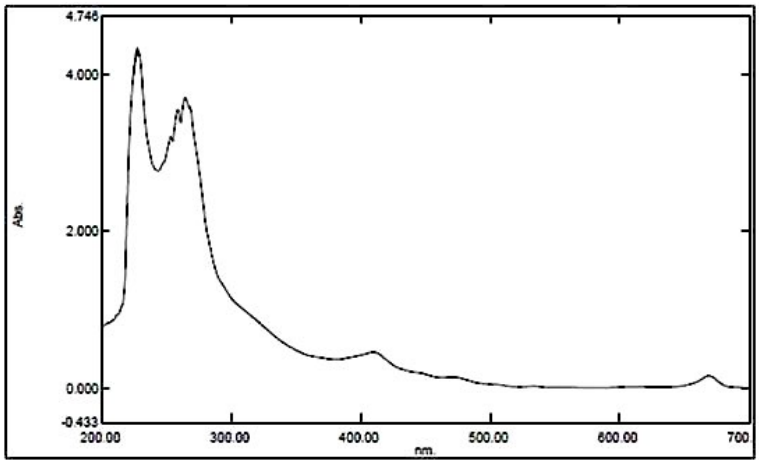
Table 2: Showing the phytochemical constituent of AQPEF (petroleum ether), AQCHF (chloroform), and AQEAF (Ethyl acetate).

Phytochemicals tests Performed		AQPEF	AQCHF	AQEAF
Alkaloids	Mayer's test	-	-	-
	Wagner's test	-	-	-
Flavonoids (Alkaline reagent test)		-	-	-
Anthraquinones (Borntrager's test)		-	-	-
Terpenoids (Kantamreddi et. al. 2010)		+++	++	+
Steroids (Kantamreddi et. al. 2010)		-	-	-
Tannins	$FeCl_3$ test	-	-	-
	Alkaline reagent test	-	-	-
	Phlobatannins (HCl test)	-	-	-
	Saponins (Froth test)	+	+	-
Glycosides (Alkaline reagent test)		-	-	-
Carbohydrates (Fehling's test)		-	+	-

3.5 Chemical characterization

3.5.1UV-Vis spectrum analysis

Data obtained from UV-Vis spectrophotometer (Shimadzu, UV 2600) shows that AQPEF has UV absorbance with the maxima at 227nm. It also shows a significant UV absorbance at 258 and 264nm (Figure 5).



No.	P/V	Wavelength	Abs.
1	P	669.00	0.149
2	P	610.50	0.015
3	P	561.00	0.003
4	P	533.00	0.020
5	P	471.50	0.132
6	P	409.50	0.454
7	P	264.00	3.702
8	P	258.50	3.555
9	P	227.00	4.314
10	V	629.50	0.006
11	V	580.00	-0.002
12	V	548.00	0.001
13	V	523.00	0.010
14	V	463.00	0.126
15	V	381.00	0.360
16	V	261.00	3.389
17	V	243.00	2.773

Figure 5: Showing the UV spectrum and the relative absorbance of AQPEF.

3.5.2 FT-IR Spectroscopy analysis

Fourier Transform Infrared Spectrophotometer (FT-IR) is the most authentic tool for the characterization of functional groups. Here, the FT-IR spectrum of AQPEF shows that it contains the major peak at 2924.09, 1732.08, 1616.35, 1454.33, 1365.60, 1247.94, 1085.92, 1008.77, 800.46 cm^{-1}which implies the presence of alkane group (C-H), aldehyde group (C=O), conjugated alkane (C=C), methyl group (C-H), alcohol group (O-H), alkyl aryl ether group (C-O), aliphatic ether / primary alcohol group (C-O), alkene group (C-C), alkene group (C-C) respectively (Figure 6).

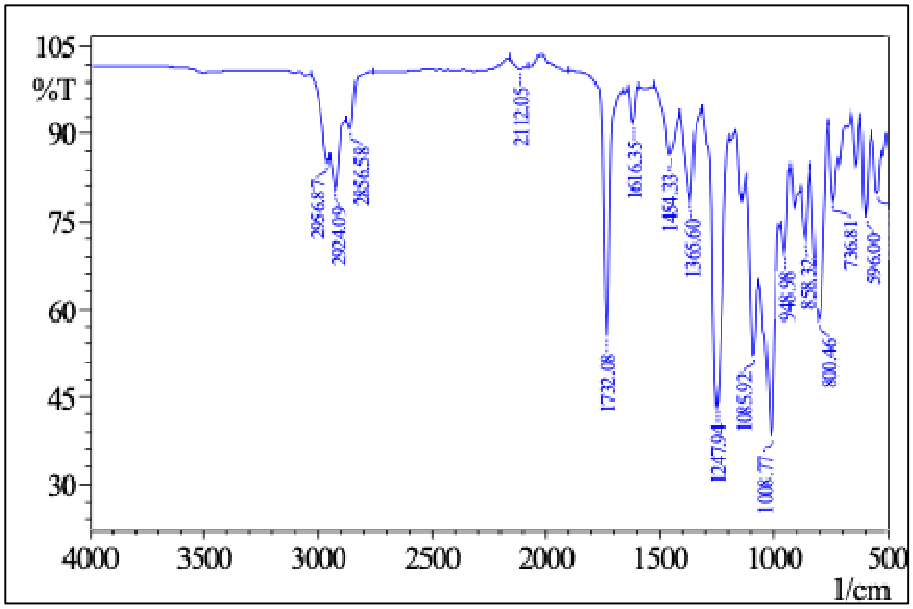


Figure 6: Showing the FT-IR spectrum of AQPEF.

3.5.3 HPLC analysis

High performance liquid chromatographic separation of AQPEF, using the C-18 column (Hitachi) and acetonitrile-water with a ratio of 70:30 as the mobile system at a flow rate of 1 mL/min, shows six main peaks with the retention time of 3.950, 4.637, 5.803, 7.440, 9.507 and 9.907 (Figure 7).

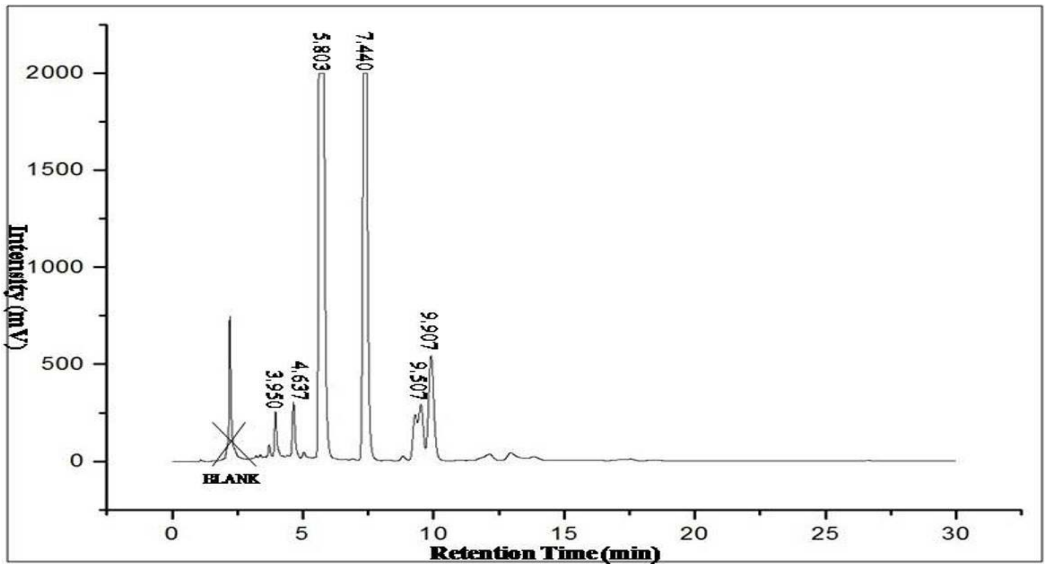


Figure 7: Showing the HPLC chromatogram of AQPEF.

3.5.4 LC-MS analysis

The liquid chromatogram of AQPEF shows that it contains two major peaks with a retention time of 1.66 and 1.74 min. The mass spectrum of the compound with a retention time of 1.66 min shows molecular ion peak [M+Na] at m/z 460 and the compound with a retention time of 1.74 shows molecular ion peak [M+Na] at m/z 458. Molecular weight obtained from the mass spectrum reveals that AQPEF contains Dihydroclerodin [M+Na, m/z 460] and Clerodin [M+Na, m/z 458] respectively (Figure 8).

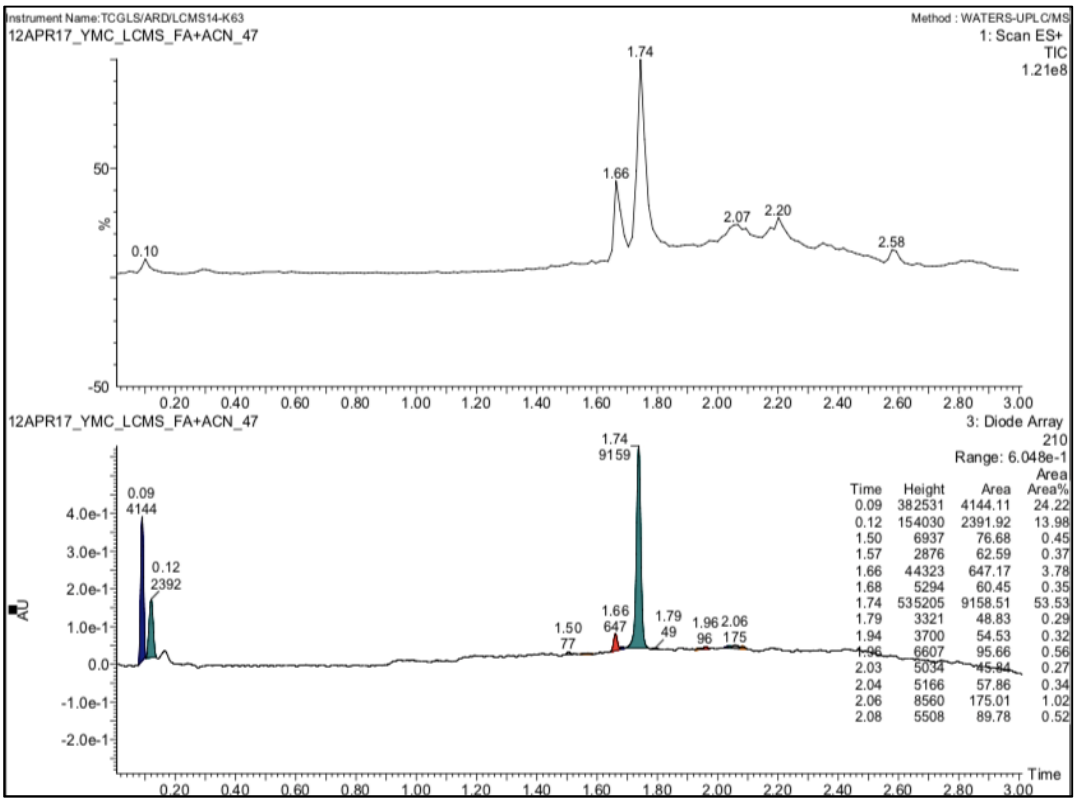


Figure 8: Showing the liquid chromatogram of AQPEF.

3.5.5 GC-MS analysis

Gas chromatogram of AQPEF indicates the presence of four (4) compounds with the retention time of 14.038, 14.103, 14.480, and 14.655 respectively. Analysis of the major peak obtained in gas chromatogram with the retention time of 14.038, recorded to have the molecular weight of **m/z 434.3** is similar to Clerodin (Mass fragments of clerodin - m/z (55, 69, 81, 95, 111,133, 145, 159, 173, 187, 204, 221, 233, 247, 264, 286, 301, 319, 321, 331,

349, 361, 374, 391, 405, 417 and **434**) (Abbaszadeh et al. 2012, Lin et al. 1989). Rest of the peaks with the retention time of 14.103, 14.480 and 14.655 has clear coordination with 15-hydroxy-14, 15-dihydroclerodin (Mass fragments m/z (55, 81, 111, 133, 175, 204, 229, 264, 286, 314, 349, 374, 406, 434 and**452**), 15-methoxy-14, 15-dihydroclerodin (Abbaszadeh et al. 2012, Lin et al. 1989, 49], 14, 15-dihydroclerodin (Mass fragments m/z (436)69, 91, 113, 133, 173, 204, 233, 263, 288, 307, 331, 351, 376, 393, 417, and **436)**(Abbaszadeh et al. 2012) (Figure 9).

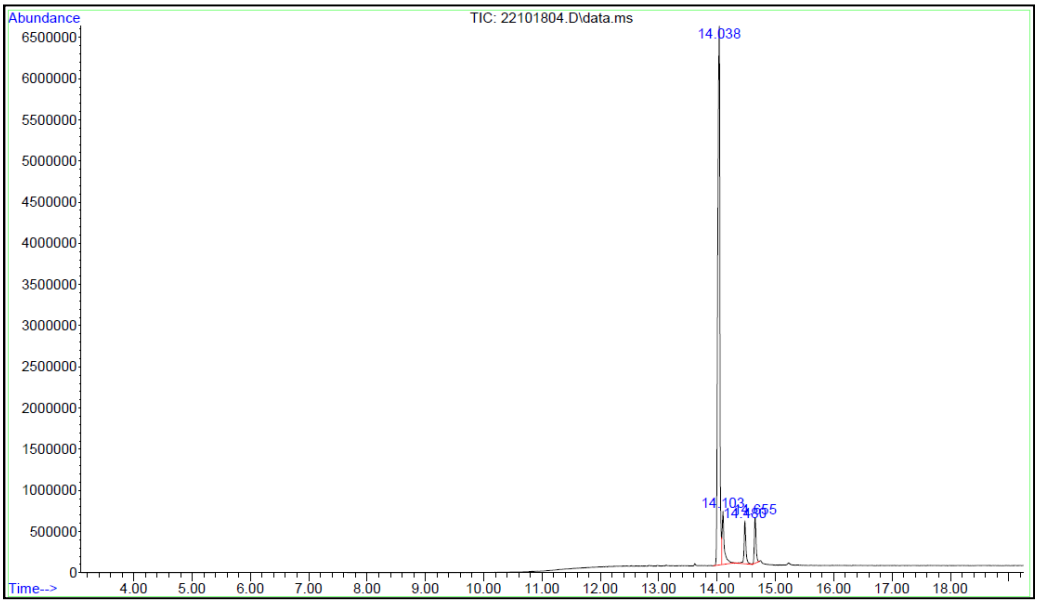


Figure 9: Gas chromatogram of AQPEF.

4. Discussion

The different extracts of *Clerodendrum viscosum* have anti-septic, anthelmintic, anti-inflammatory, anti-leprosy, anti-pyretic, anti-tumor, anti-bacterial, antiproliferative, metaphase arresting, apoptosis-inducing, antifeedant activities, *etc*. (Yusuf et al. 1994, Warrier et al. 1996, Jirovetz et al 1999, Aley et al. 2011, Ray et al. 2012, Kundu and Ray, Ray et al. 2013, Gouthamchandra et al. 2010). This study is in continuous with the previous investigation that indicated antiproliferative, metaphase arresting, mitotic abnormality, and micronuclei inducing potentials of leaf aqueous extract of *C. viscosum* (LAECV) (Kundu and Ray 2016, Ray et al. 2013). The focus of the present investigation was to isolate and identify the active principle(s)

responsible for cell cycle delay, metaphase arrest and c-metaphase induction in *A. cepa* root apical meristem cells.

The LAECV was successively fractionated with petroleum ether (AQPEF), chloroform (AQCHF), and ethyl acetate (AQEAF). The *A. cepa* root growth retardation analysis indicates that petroleum ether fraction (IC50 = 23.68 ± 5.5 µg/mL) has more root growth retardation/antiproliferative activity than chloroform (IC50 = 62.78 ± 3.26 µg/mL) and ethyl acetate fraction (IC50 = 106.15 ± 4.03 µg/mL). Many authors suggest that antiproliferative and cytotoxic effects of plant extracts or chemical substances can be evaluated using *A. cepa* root tip cells and it is very commonly used in the assessment and monitoring of environmental toxicants (Bakare et al. 2000, 2012, 2013, Levan 2010, Frescura et al. 2012). The percentage of root growth retardation depends on the antiproliferative potentials of the treated substances. The AQPEF treatment for 4 h followed by 16 h recovery shows a dose-dependent increase in *A. cepa* root apical meristem swelling. The AQPEF induced root swelling both in *A. cepa* root and *T. aestivum* seedlings, indicating the metaphase arresting bioactive principles that were extracted mainly in petroleum ether fraction of LAECV. It can be assumed that the generation of polyploidy is related to the root apical meristem swelling in AQPEF treatment because of the cellular dimension increases in polyploid cells. The presence of terpenoids in LAECV and its successive fractions indicate that terpenoids may be responsible for the root growth retardation effect (Ray et al. 2012). Our previous investigation indicated that LAECV has colchicine-like metaphase arrest and mitotic abnormalities inducing potentials (Ray et al. 2012, Kundu and Ray 2016) and this study indicates that the successive petroleum ether fraction (AQPEF) of LAECV contains the active principles responsible for metaphase arrest and c-metaphase. Earlier we observed a similar root swelling patterns in seedlings after colchicine and LAECV exposure (Ray et al. 2013). Investigation by squash preparation of *A. cepa* root apical meristem cells revealed that AQPEF induced increased metaphase frequencies in a dose-dependent manner at both 2 h and 4 h treated samples indicate that AQPEF has definite metaphase arresting activity. The AQPEF treatment for 2 h and 4 h in *A. cepa* root tip cells result in decreased frequencies of prophase, anaphase, and telophase in a dose-dependent manner. Subsequently, the frequencies of prophase, anaphase, and telophase showed an increasing tendency in recovery root tip cells (4+16 h) as compared to 2 h and 4 h treated cells. The AQPEF treatment for 2 h and 4 h showed increased MI%, whereas it decreased in 4 h treatment followed by 16 h recovery root tip cells.

The probable reason for mitotic index elevation in 2 h and 4 h treated cells may be due to an increase in metaphase frequencies (Davidson et al. 1966). Similarly, reduction in metaphase frequencies and cytotoxicity exerted by the AQPEF may lead to mitotic index depression in 4 h treatment followed by 16 h recovery in *A. cepa* root tip cells.

Further investigation shows that the AQPEF induces c-metaphase at 2 h, and 4 h treatment and 4 h treatment + 16 h recovery in *A. cepa* root tip cells. The formation of c-metaphase, colchicine-like metaphase, is directly correlated with the microtubule disruption and indicates that phytochemicals present in AQPEF may have colchicine-like microtubule destabilizing activity (FiskesjÖ 2008, Bonciu et al. 2018, Shahin and El-Amoodi 1991). These data also correlate with the occurrence of c-metaphase in LAECV and colchicine treated *A. cepa* root tip cells (Kundu and Ray 2016). The formation of c-metaphases may result from the spindle poisoning effect of microtubule destabilizing drugs or chemicals (FiskesjÖ 2008, Bonciu et al. 2018). Correlation between the formation of c-metaphase, vagrant chromosome, and polyploidy was evident by many investigators in *A. cepa* root tip cells (Carvalho et al. 2019, Levan 2010). Based on this correlative evidence, we can postulate that AQPEF has a similar effect on *A. cepa* root tip cells.

The HPLC analysis of AQPEF depicted six peaks with two large peaks at the retention time of 5.803 and 7.440 min. The LC-MS analysis revealed that AQPEF contains two major compounds; Dihydroclerodin (M+Na, m/z 460, Rt-1.66 min) and clerodin (M+Na, m/z 458, Rt-1.74 min) having the retention time of 1.66 and 1.74 min respectively. Further investigation by GC-MS analysis revealed the presence of clerodane diterpenoids like clerodin (m/z 434.3), 15-hydroxy-14, 15-dihydroclerodin (m/z 452), 15-methoxy-14, 15-dihydroclerodin (m/z 466), and 14, 15-dihydroclerodin (m/z 436) with the retention time of 14.038, 14.103, 14.480 and 14.655 respectively (Abbaszadeh et al. 2012, Lin et al. 1989). Investigation of AQPEF by UV-Vis spectrophotometer and FT-IR shows that petroleum ether fraction has absorption maxima at 227 nm and contains alkane group (C-H), aldehyde group (C=O), conjugated alkane (C=C), methyl group (C-H), alcohol group (O-H), alkyl aryl ether group (C-O), aliphatic ether / primary alcohol group (C-O), alkene group (C-C) (Lin et al. 1989) which are in agreement with the obtained data of LC-MS and GC-MS. A previous investigation by various authors also stated that aerial parts of the *C. viscosum* contain clerodin and many other clerodane diterpenoids (Abbaszadeh et al. 2012). The large group of clerodane diterpenes occurs naturally as secondary metabolites in

several hundreds of plant species from various families and in organisms from other taxonomic groups, such as fungi, bacteria, and marine sponges. In recent years, there is increasing attention on clerodane diterpenoids because of their noteworthy biological activities, particularly as insect antifeedant against economically important insect phytophagous pests. The various genera of the Lamiaceae family have been identified as rich sources of antifeedant clerodane diterpenoids. The presence of metaphase arresting, and c-metaphase inducing capabilities of both LAECV and AQPEF and also the presence of clerodane diterpenoids in AQPEF indicate that clerodane diterpenoids of LAECV may be the active principles for metaphase arrest, c-metaphase, and cell cycle delay.

5. Conclusion

The present study was conducted to identify the chemical compositions of LAECV that are responsible for cell cycle delay, pro-metaphase arrest, and c-metaphase induction in *A. cepa* root apical meristem cells. Fractionation and bioassay data indicate that petroleum ether fraction, AQPEF, is the main bioactive fraction containing clerodane diterpenoids like clerodin (m/z 434.3), 15-hydroxy-14, 15-dihydroclerodin (m/z 452), 15-methoxy-14, 15-dihydroclerodin (m/z 466), and 14, 15-dihydroclerodin (m/z 436). Further detailed investigation is required for purification of the individual clerodane diterpenes of *C. viscosum* and to test their relative pro-metaphase arrest and mitotic abnormality inducing potentials as well as a comparative analysis with the colchicine actions.

Disclosure statement

No conflict of interest was declared.

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