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E. coli AB 1157 susceptibility test, MTT assay on MCF-7 and HeLa cell lines of root and leaf fractions of *Viburnum* Linn. species

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The aim of this study was to evaluate mutagenic bacterial susceptibility and cytotoxic potency of *Viburnum coriaceum* Blume and *Viburnum erubescens* Wall.ex DC in order to report the actual chemotherapeutic potentials of these two species. The methanolic (80%) leaf and chloroform root extracts of *Viburnum* Linn. Species were tested for their bacterial strain based cytotoxicity employing Agar diffusion method suing *E. coli* AB 1157 strain. Also, the MTT assay was carried out employing MCF-7 breast cancer cell lines and HeLa cervical cell lines. It was started with IC_{50} value determination of the selected test extracts from the results of bacterial strain based cytotoxicity. Upon increase in concentration up to 1000 µg/mL in agar diffusion cytotoxicity studies VCMLE, VEMLE and VCCRE had shown diameter of inhibition zone 10 mm, 9 mm and 10 mm respectively. Among other extracts, the VEMLE and VCCRE were selected to go ahead with anticancer activity by MTT assay. The potentials of extracts through cytotoxicity mechanism had produced 30-40% protection against cancer cell lines. It was concluded that VEMLE and VCCRE had produced the cytotoxic effect on *E. coli* AB 1157 strain. and were selected for the cytotoxic studies. The effects exhibited by the selected extracts may be due to the presence of diverse number of active constituents present in *Viburnum* Linn species also may be to the presence of unreported constituents.

Keywords: Cytotoxicity, HeLa, MTT assay, MCF-7, Susceptibility test, *Viburnum*

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The genus, *Viburnum*, formerly belonging to Caprifoliaceae and in recent years positioned under Adoxaceae family, contains about 200 species throughout the world[1,2], and about 17 species of them have been reported in India. The *Viburnum* have been reported in literature to possess uterine sedative, anti-asthmatic, astringent, anti-inflammatory and anti-microbial activities[3,4]. A verbal enquiry to the tribal of Ooty and Coimbatore, Tamilnadu, also supported that aforesaid pharmacological activities were traditional and were promising with roots and leaves of these species[5].

And a qualitative chemical screening to reveal that the leaf part of these two species contains an appreciable amount and a wide range of phenolic compounds[6,7]. A few of these species have been reported to contain bio-active molecules such as: triterpenes phytosterols, (pentacyclic) and phenolic compounds tannins, flavonoids, anthocyanidins, iridoid glycosides, biflavones, phenolic compound of C6-C3 skeletons and their glycosides. Even though the genus is popular for its big population, the number of species scientifically investigated and the volume of research establishments on records are notably scant, especially on their pharmacological behaviours. Therefore, it has been decided to select some two species of the genus-*Viburnum* Linn., To begin with the current study, different solvent extracts of the two species were subjected to a preliminary phyto-chemical investigation to select appropriate solvent extracts.

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Based on the type of phyto-constituents detected in the selected extracts deduced from preliminary phyto-chemical analysis and informations reported in literatures. In order to obtain reliable results, suitable methods were selected for evaluation. The selected plant species and chemotherapeutic study carried out on these also were very scant. Therefore, the result of the present study will definitely be the good start and lead for the further investigations.

Materials and Methods

Collection of specimens

The leaf and roots of *Viburnum* Linn. Species were collected from Nilgiri hills at an altitude of 1500–1800 ft, were authenticated by Dr Chelladurai, (Ex Professor) medicinal plants supply for siddha, Govt. of India, Tamilnadu, to undertake some pharmacological investigations. The voucher specimens of *Viburnum coriaceum* Blume and *Viburnum erubescens* Wall.ex DC were labelled (VC131) and (VE131), and deposited in the department of pharmacognosy at Cherraan's College of Pharmacy, Coimbatore, Tamil Nadu. The photograph of the plant species taken at the location-Nilgiri Hills were also submitted (Fig. 1 & Fig. 2).

Bacterial strain based cytotoxicity screening by Agar diffusion method

Bacterial Strain Based Cytotoxicity was carried out using Agar diffusion method and the sample was prepared using DMSO (dimethyl sulfoxide). The stock solution was 10 mg/mL sample in DMSO. Extracts to be screened were prepared in various concentrations such as 25, 50, 100, 250, 500 and 1000 µg/mL. Then the media for the cytotoxicity study was prepared to contain Tryptone-10 g, NaCl-10 g and Yeast extract 5 g, Agar 20 g in 1000 mL of distilled water. The *E. coli* AB 1157, a wild-type strain, proficient to repair damage in the DNA is considered for this study. Initially, the stock culture of bacteria was revived by inoculating in broth medium and grown at 37ºC for 18 h. The LB Agar plates were prepared and wells were made in the solidified LB agar plate. Each plate was inoculated with 18 h old cultures (100 µL, 10-4 cfu) and spread evenly on the plate. After 20 min the wells were filled with compound at different concentrations. All the plates were incubated at 37ºC for 24 h and the diameter of inhibition zone were noted[8].

Evaluation of cytotoxicity by MTT assay method

This is a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The cells are then solubilized with an organic solvent (e.g., DMSO, Isopropanol) and the released, solubilized formazan reagent is measured colorimetrically. Since the reductions of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells[9,10,11].

Fig. 1 — *Viburnum coriaceum* plant at the location-Nilgiri Hills

Fig. 2 — *Viburnum erubescens* plant at the location-Nilgiri Hills

Effect of extracts on HeLacervical cancer cell lines and MCF-7 breast cancer cell lines

For the MTT assay method Human HeLa cervical cancer cell lines were procured from Biogenics Lab Bangalore. The cell line were cultured in DMEM (Dulbecco's Modified Eagle Medium) (Cat No-11965-092) medium which was supplemented with 10% heat inactivated FBS (Fetal Bovine Serum) (Gibco, Invitrogen) Cat No-10270106, and 1% Antibiotic–Antimycotic 100X solution (containing 10000 U/mL of penicillin, 10000 µg/mL streptomycin and 25 µg/mL of Gibco amphotericin-B) (Thermofisher Scientific)-Cat No-15240062 .

The cells were seeded at a density of approximately 5×103 cells/well in a 96-well flat-bottom micro plate and maintained at 37°C in 95% humidity and 5% CO_2 for overnight. Different concentration (200,100, 50, 25, 12.5, 6.25 µg/mL) of samples was treated. The cells were incubated for another 48 h. The cells in well were washed twice with phosphate buffer solution, and 20 µL of the MTT staining solution (5 mg/mL in phosphate buffer solution) was added to each well and plate was incubated at 37°C. After 4 h, 100 µL of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals, and absorbance was recorded with a 570 nm using micro plate reader[12,13,14].

For the estimation of surviving cells, the below mentioned formula was used:

Mean OD of test compound

$$\text{Surviving cells (\%)} = \frac{\text{Mean OD of Negative control}}{\text{Inhibiting cells (\%)} = (100 - \text{Surviving cells})} \times 100$$

Results

For the Bacterial Strain Based Cytotoxicity study extracts such as VCMLE, VEMLE, VCCRE and VECRE were selected and various concentrations (25 µg/mL, 50 µg/mL, 100 µg/mL, 250 µg/mL, 500 µg/mL, 1000 µg/mL) were also made. After the incubation period of 24 h at 37°C, the diameter of inhibition was analyzed. No extract had prevented the growth of bacterial strain up to the concentration 250 µg/mL. At 500 µg/mL concentration VCMLE and VCCRE showed 5 mm and 3 mm zone of inhibition respectively against the *E. coli* AB1157 strain whereas no inhibition exhibited by the VEMLE and VECRE (Table 1 & Fig. 3).

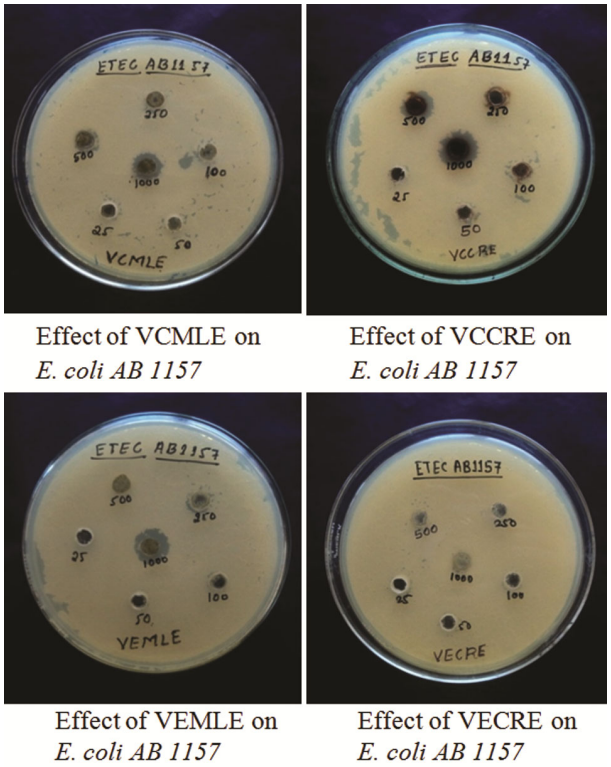
The cytotoxicity studies were started with IC_{50} value determination and it was to be 562.17±25.14 µg/mL of VCMLE against the MCF-7 cell lines whereas against the HeLa cell lines it was 358.02±12.45 µg/mL.

The chloroform extract showed an IC_{50} value as 759.90±31.20 µg/mL against the growth of MCF-7

Table 1 — Effect of extracts on bacterial strain based carcinogenicity

Extracts	25 µg	50 µg	100 µg	250 µg	500 µg	1000 µg	MIC µg
VCMLE	0	0	0	0	5	10	500
VEMLE	0	0	0	0	0	9	1000
VCCRE	0	0	0	0	3	10	500
VECRE	0	0	0	0	0	0	NF

Values presented are diameter of inhibition zones in mm and MIC-Minimum Inhibitory Concentration, NF-Not Found, VCCRE- *V. coriaceum* chloroform root extract, VECRE- *V. erubescens* chloroform root extract, VCMLE- *V. coriaceum* methanolic leaf extract, VEMLE- *V. erubescens* methanolic leaf extract



Effect of VCMLE on *E. coli AB 1157* | Effect of VCCRE on *E. coli AB 1157*

Effect of VEMLE on *E. coli AB 1157* | Effect of VECRE on *E. coli AB 1157*

Fig. 3 — Bacterial Strain Based Cytotoxicity Screening of *Viburnum* Linn Extracts Values 25, 50, 100, 250, 500, 1000µg/mL concentration of test sample studied; ETEC- Enterotoxic *E. coli* AB1157, VCCRE- *V. coriaceum* chloroform root extract, VECRE- *V. erubescens* chloroform root extract, VCMLE- *V. coriaceum* methanolic leaf extract, VEMLE- *V. erubescens* methanolic leaf extract

breast cancer cell lines whereas the same extracts exhibited 433.20±15.33 µg/mL as IC_{50} against the HELA cervical cancer cell lines (Table 2).

Then the cell viability assay was carried out using various concentrations of the extract such as 200 µg/mL, 100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL and 6.25 µg/mL. The maximum viability (99.67±4.66%) retained in case of VCCRE at 6.25 µg/mL, whereas, the highest inhibition of growth of cell lines (29%) was achieved at 200 µg/mL. The VCMLE had produced maximum inhibition (36%) at 200 µg/mL whereas the least inhibition (5%) at 6.25 µg/mL. The result revealed that the VCMLE had been slightly more potent than VCCRE against MCF-7 cell lines (Table 3, Fig. 4 & Fig. 5). The HELA cervical cancer cell lines were involved in cell viability analysis using concentrations as in the case of MCF-7 cell lines. The VCCRE showed slightly improved inhibition (35%) at 200 µg/mL concentration whereas the extract could only produce inhibition of cell growth around 3% at 6.25 µg/mL concentration. As like VCCRE, VCMLE also had shown somewhat improved protection against HELA cancer cells i.e., around 40% inhibition and 60% retention of viability whereas, at 6.25 µg/mL concentration it showed around 4% protection (Table 4, Fig. 6 & Fig. 7).

Table 2 — IC_{50} value of VCMLE and VCCRE in µg/mL concentration on HeLa and MCF-7 cancer cell lines

Extracts	IC_{50} (µg/mL)	
	MCF-7	HeLa
VCMLE	562.17±25.14	358.02±12.45
VCCRE	759.90±31.20	433.20±15.33

Values are expressed as mean±SEM, n=3, VCCRE- *V. coriaceum* chloroform root extract, VCMLE- *V. coriaceum* methanolic leaf extract

Table 3 — Cell viability analysis of VCMLE and VCCRE on MCF-7 Breast cancer cell lines

Concentration	MCF-7 Cell lines Viability (%)	
	VCMLE	VCCRE
200 µg/mL	65.68±4.51	71.77±2.89
100 µg/mL	71.36±3.70	76.54±4.03
50 µg/mL	79.84±2.93	85.76±4.24
25 µg/mL	85.38±2.26	86.98±2.54
12.5 µg/mL	89.43±4.04	93.87±5.16
6.25 µg/mL	94.97±4.47	99.67±4.66

Values are expressed as mean±SD, n=3, VCCRE- *V. coriaceum* chloroform root extract, VCMLE- *V. coriaceum* methanolic leaf extract

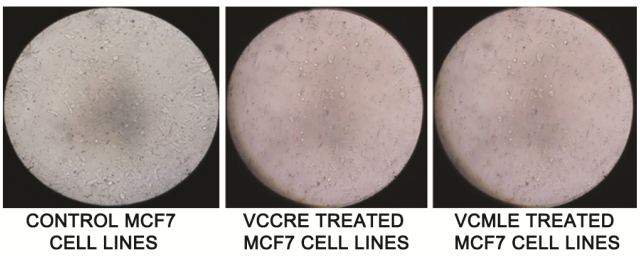


Fig. 4 — Cell viability analysis of VCMLE and VCCRE on MCF-7 cancer cell lines VCCRE- *V. coriaceum* chloroform root extract, VCMLE- *V. coriaceum* methanolic leaf extract

Discussion

The methanolic Leaf and chloroform root extracts were tested for their bacterial strain based cytotoxicity employing Agar diffusion method. For the analysis *E. coli* AB1157, a wild-type strain was selected because of their capacity to repair the damage caused to their DNA.

Also, the literature reviews of *E. coli* AB1157 (a wild-type, K-12 strain having no known defects in DNA repair capability) had shown unusual resistance when irradiated daily with very large X-ray doses and

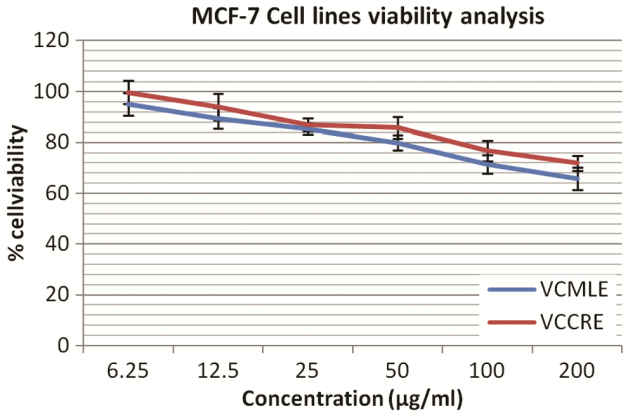


Fig. 5 — Cell viability analysis of VCMLE and VCCRE on MCF-7 cancer cell lines VCCRE- *V. coriaceum* chloroform root extract, VCMLE- *V. coriaceum* methanolic leaf extract

Table 4 — Effect of VCMLE and VCCRE on HeLacervical cancer cell lines

Concentration	HeLaCell lines Viability (%)	
	VCMLE	VCCRE
200 µg/mL	60.36±2.12	64.41±3.01
100 µg/mL	69.13±4.22	71.67±4.91
50 µg/mL	75.63±.3.74	79.36±2.48
25 µg/mL	83.03±4.01	86.74±4.07
12.5 µg/mL	89.76±5.14	92.87±5.11
6.25 µg/mL	96.35±3.22	97.04±3.24

Values are expressed as mean±SD, n=3, VCCRE- *V. coriaceum* chloroform root extract, VCMLE- *V. coriaceum* methanolic leaf extract

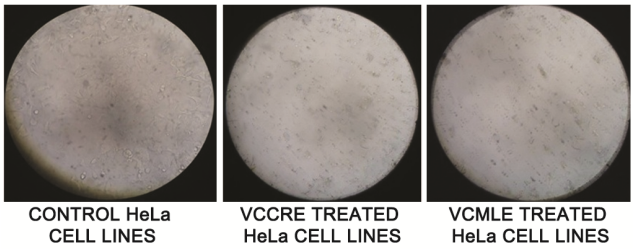


Fig. 6 — Effect of VCMLE and VCCRE on HeLa Cervical Cancer Cell lines-% Cell Viability Analysis VCCRE- *V. coriaceum* chloroform root extract, VCMLE- *V. coriaceum* methanolic leaf extract

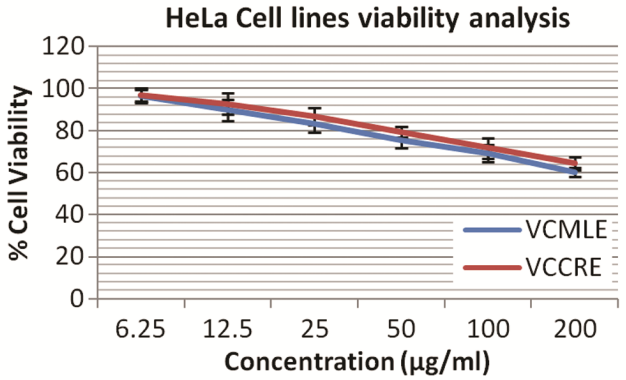


Fig. 7 — Cell viability analysis of VCMLE and VCCRE on HeLa cancer cell lines VCCRE- *V. coriaceum* chloroform root extract, VCMLE- *V. coriaceum* methanolic leaf extract

UV photons[15]. The resistance exerted by the species compelled us to select *E. coli* AB1157 for the susceptibility study. Due to its high resistance, the selected extracts- VCMLE, VEMLE, VCCRE and VECRE were expected to show either very little or no effect.

In the susceptibility study only when concentration was increased up to 1000 µg/mL VCMLE, VEMLE and VCCRE had shown diameter of inhibition zone 10 mm, 9 mm and 10 mm respectively. The only extract VECRE did not show MIC in any of the concentrations tested and suggests that these compounds did not exhibit any deleterious effect or toxicity to the bacteria. The susceptibility study further confirms that the extracts selected for the current study are free from either of mutagenicity, carcinogenicity and cytotoxicity effects.

The extracts VCMLE and VCCRE were selected for the anticancer studies as the cytotoxicity studies result suggested these would be effective. The anticancer activity was performed using MTT (3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay method and the formation of formazan measured colorimetrically.

Before MTT assay IC_{50} value determination of the selected test extracts against the MCF-7 breast cancer cell lines and HeLa cervical cell lines was done. MCF-7 breast cancer cell line (Michigan Cancer Foundation-7) and HeLa cervical cancer cell line (named after the first victim Henrietta Lacks) were possessing estrogen and progesterone receptors, and estrogen receptor respectively. The cytotoxicity potentials exhibited by the extracts may be due to the presence of diverse number of phytoconstituents[16] which were already reported and unreported in the literatures. Also, the effect believed to be the affinity of *Viburnum* extracts towards the estrogen and progesterone receptors needs further investigation[17].

Finally, result suggested that both the extracts performed very closely against cancer cell lines but VCMLE dominated VCCRE extract in higher concentrations.

Conclusion

From the result of the study it was concluded that VECRE did not produce observable ZOI on *E. coli* AB1157 bacterial strain and presumed to be not having any cytotoxic effect. But the other three extracts had produced comparable cytotoxic effects at the level of 1000 µg/mL concentration level gave a clue to proceed with anticancer activity by MTT assay method. The MTT assay further concluded that anticancer potentials of extracts through cytotoxicity mechanism had produced pronounced effect about 30-40% protection at 200 µg/mL concentration was good enough to try with more concentrations. From the result of these studies, the cytotoxic effect it is assumed that it may be due to the presence of diverse number of active constituents present in *Viburnum* Linn species, also, may be the phytoconstituents and their affinity towards the estrogen and progesterone receptors present on the cancer cell lines. Apart from these two species among 17 species reported in India, needed to be thoroughly analysed for their anticancer potentials and the result drawn from the current study will definitely be useful lead.

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