Research Article**Antimicrobial and anticancer activities of ethanol and methanol extracts of wild and micropropagated *Cadaba fruticosa* (L.) Druce****Yessian Sharmila Juliet, Kandasamy Kalimuthu*, Vajjiram Chinnadurai, Venkatachalam Ranjitha, Ammasai Vanitha***Plant Tissue Culture Division, PG and Research Department of Botany, Government Arts College (Autonomous), Coimbatore-641018, India.*

Received: 9 August 2018

Revised: 28 August 2018

Accepted: 16 September 2018

Abstract

Objective: Ethanol and methanol extracts of wild and tissue cultured *Cadaba fruticosa* were studied and compared for its antimicrobial activity against six human pathogenic organisms and anticancer activity against HeLa cancer cell lines. **Materials and Methods:** Antimicrobial activity of Wild *Cadapa fruticosa* ethanol, Micropropagated/ tissue cultured plant ethanol, Wild *Cadapa fruticosa* methanol, Micropropagated/tissue cultured plant methanol (WCFE, MCFE, WCFM and MCFM) plant extracts were investigated by well diffusion susceptibility method and also *in vitro* cytotoxicity activity was studied by MTT assay at different concentration. **Results and Conclusion:** The results showed that the highest zone of inhibition was obtained in *Escherichia coli* (14 ± 0.82 mm and 08 ± 1.05 mm) at 60 μ l concentration of wild and tissue cultured *Cadaba fruticosa* ethanol extracts. Whereas in methanol extract the highest zone of inhibition (14 ± 0.14 mm and 09 ± 0.12 mm) in 60 μ l concentration against *Streptococcus pyogenes* and *Staphylococcus aureus*. In both the cases the activity of the extract was less against fungal pathogens. The higher percentage of anticancer activity was observed in wild and tissue cultured *Cadaba fruticosa* of ethanol extracts with 53.14 and 54.78 at 5 mg/ml concentration. Whereas in wild and tissue cultured *Cadaba fruticosa* methanol extracts treatment were 52.58 and 51.39 respectively. The percentage of inhibition was increased with the increasing concentration. The IC_{50} value of wild and tissue cultured *Cadaba fruticosa* ethanol extracts were 260.36 mg/ml and 268.05 mg/ml, methanol extracts were 273.71 mg/ml and 286.73 mg/ml respectively. Results of this study are important because these results confirmed the use of tissue cultured plants instead of natural plants. However more investigation are needed to identify the active principles and its molecular mechanism to explain their therapeutic efficacy.

Keywords: *Cadaba fruticosa*, *Streptococcus*, HeLa cancer, tissue cultured plant

Introduction

Cancer is the prominent cause of death in economically developed countries and the second leading cause of death in developing countries (Jemal et al., 2011). Indians are mostly affected by cancer due to factors such as Urbanization, industrialization, lifestyle changes and population growth. Further, increased life span has led to increase cancer since the incidence of cancer rises with age. India has reported over 1.1 million new cases in the year 2015 and the number is expected to be around 2.1 million by the year 2020 (Ali et al., 2011).

Chemotherapy is one of the most frequently used therapeutic modalities for the treatment of cancer (Li et al., 2009; Shengtao et al., 2012). But its severe side effects due to toxicity leads to search for an alternative and complementary medicine from natural plant, without or less side effects.

Cadaba fruticosa (L.) Druce is a medicinally important shrub or a small tree, belonging to the family Cappariaceae and it is used in Indian traditional medicinal system. The leaf juice is internally used in the case of general weakness, energetic during dysentery, diarrhoea, also to relieve general body pain, antidote against poisoning, stimulant, and antiscorbutic (Provitamin, 1975; Sandhya et al., 2006). Indian *Cadaba*, the Indian medicinal plant finds usage in various chronic ailments, known to be effective for prolonged periods. The leaves and roots are considered deobstruent, anthelmintic and emmenagogue and are prescribed in the form of a

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DOI: <https://doi.org/10.31024/ajpp.2018.4.6.23>

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decoction for treating uterine obstructions. The leaves of Indian *Cadaba* are also used as a poultice to promote healing of sores. In Siddha, the leaf and fruit are used to treat worm infestation, swellings, eczema and constipation. Advances in biotechnology, especially *in vitro* culture techniques and molecular biology, provides some important tools for conservation and management of plant genetic resources. Plant tissue culture is an alternate method of commercial propagation and is being used widely for the commercial propagation of a large number of plant species, including many medicinal plants. Cultivation of medicinal plants is also difficult due to the lack of proper agronomic practices for the most species and unavailability of source plant material. *In vitro* technique has the unique advantage of propagation of the desired taxon, independent of season, reproductive barriers, germination hurdles and so on (Anuradha and Pullaiah, 2001). In fact *in vitro* propagation and cryopreservation of medicinal plants help us to conserve biodiversity. This plant is endemic to Indian sub-continent and distributed throughout the tropical and subtropical and regions of the World (Anonymous, 2005; Amudha and Rani, 2014). Numerous religions and medicinal temple trees in India, which represents its esthetic value, help in germplasm conservation. The leaves are used in the treatment of boil, cough, fever, dysentery, rheumatic pain and an ant donate against poisoning (Nadkarni, 1985).

Moreover the plant also have the active constituents, i.e, cadabicine, cadabicine diacetate (Viqar Uddin et al., 1987), Capparisine and α – B – dihydroferulic acid (Aziz-Ur-Rehman, 1990). The present study was carried out to analyze antimicrobial and anticancer activity of wild plant leaf and tissue cultured plant ethanol and methanol extracts of *Cadaba fruticosa*.

Materials and Methods

Plant collection and authentication

The plant twig with flower of *Cadaba fruticosa* were collected from the foothills of Kuppepalayam, Western Ghats, Coimbatore, Tamilnadu, India and identified by Botanical Survey of India. Plant identification reference number is BSI/SRC/5/23/2015/Tech/931 (Figure 1.A).

Chemicals and apparatus

Ethanol, methanol, nutrient broth, potato dextrose agar, petroleum ether, chloroform, ethyl acetate (AR grade). All solvents purchased and used through the study were of analytical reagent grade were supplied by Hi-media Bombay, India.

Extract preparation

Wild plant leaf (Figure 1. A) and tissue cultured plant (Figure 1. B) powder (100 gram each samples) of *Cadaba fruticosa* was packed into the thimble of Soxhlet apparatus and subjected to

extraction sequentially with petroleum ether, chloroform, ethyl acetate, ethanol and methanol. Each plant extract collected and evaporated in room temperature was stored under 4 °C. Finally ethanol and methanol extracts were used for further antimicrobial and anticancer studies.

A– Wild plant

B - Tissue culture plant

Antimicrobial activity

The test organisms used were human bacterial pathogen viz., *Streptococcus pyogenes* (MTCC442), *Staphylococcus aureus* (MTCC96), *Escherichia coli* (MTCC1195) and *Klebsiella pneumoniae* (MTCC109) and fungal pathogens like *Candida albicans* (MTCC3017) and *Trichoderma viride* (MTCC167). The bacterial and fungal cultures were maintained on nutrient broth (NB) at 37 °C and fungus was maintained on potato dextrose agar (PDA) at 28 °C.

Preparation of inoculum

The gram positive bacteria *Streptococcus pyogenes*, *Staphylococcus aureus* and gram negative bacteria *Escherichia coli*, *Klebsiella pneumoniae* were pre-cultured in nutrient broth overnight in a rotary shaker at 37 °C, centrifuged at 10,000 rpm for 5 min, pellet was suspended in double distilled water and the cell density was standardized spectrophotometrically (A_{610} nm). The fungal inoculums *Candida albicans*, *Trichoderma viride*, were prepared from 5 to 10 day old culture grown on Potato dextrose agar medium. The Petri dishes were flooded with 8 to 10 ml of distilled water and the conidia were scraped using sterile spatula.

Agar disk diffusion assay

The agar disk diffusion assay has been used as a preliminary screening for antimicrobial activity of wild and tissue culture plant ethanol and methanol extracts (Ncube et al., 2008). The dried extracts were dissolved in their respective

extracting solvents, yielding a stock solution of 1 g/ml from which various extract concentrations were prepared by dilution. Sterile 9 mm disks were impregnated with various concentrations like 20 µl, 40 µl and 50 µl of wild and tissue culture plant ethanol and methanol extracts and incubated at 37 °C for 24 hrs. to dry. Each extract was tested in triplicate. The positive control substances as antibiotic of Streptocycline. The dried sterile disks were then placed carefully onto the surface of the agar inoculated with microbial culture. The inhibition zones were measured in millimeters (mm) from the circumference of the disk recorded (Salie et al., 1996). The activity of bacterial pathogen was determined after 24 hrs. at 37 °C and also fungal pathogen was determined after 72 hrs. of incubation at 28 °C.

Cytotoxicity study

Cytotoxicity of wild plant leaf and tissue cultured plant ethanol and methanol extracts were studied through HeLa carcinoma cell by MTT assay (Mosmann, 1983).

Cell line

The human cervical cancer cell line (HeLa) was obtained from National Centre for Cell Science (NCCS), Pune and grown in Eagles Minimum Essential Medium containing 10% fetal bovine serum (FBS). The cells were maintained at 37 °C, 5% CO₂, 95% air and 100% relative humidity. Maintenance cultures were passaged weekly, and the culture medium was changed twice a week.

Cell treatment procedure

The monolayer cells were detached with trypsin, Ethyldiamine tetra acetic acid (EDTA) to make single cell suspensions and viable cells were counted using a hemocytometer and diluted with medium containing 5% FBS to give final density of 1x10⁵ cells/ml. One hundred microliters per well of cell suspension were seeded into 96-well plates at plating density of 10,000 cells/well and incubated to allow for cell attachment at 37 °C, 5% CO₂, 95% air and 100% relative humidity. After 24 hrs. the cells were treated with serial concentrations of the test samples. They were initially dissolved in dimethyl sulfoxide (DMSO) and an aliquot of the sample solution was diluted to twice the desired final maximum test concentration with serum free medium. Additional four serial dilutions were made to provide a total of five sample concentrations. Aliquots of 100 µL of these different sample dilutions were added to the appropriate wells already containing 100 µL of medium, resulting in the required final sample concentrations. Following sample addition, the plates were incubated for an additional 48 hrs. at 37 °C, 5% CO₂, 95% air and 100% relative humidity. The medium containing without samples were served as control and triplicate was maintained for all concentrations.

MTT assay

3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyltetrazolium bromide (MTT) is a yellow water soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells.

After 48 hrs. of incubation, 15 µL of MTT (5 mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37 °C for 4 hrs. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100 µl of DMSO and then the absorbance was measured at 570 nm using micro plate reader. The percentage cell viability was then calculated with respect to control as follows.

$$\% \text{ Cell viability} = [\text{A}] \text{ Test} / [\text{A}] \text{ control} \times 100$$

$$\% \text{ Cell inhibition} = 100 - [\text{A}] \text{ Test} / [\text{A}] \text{ control} \times 100$$

Statistical Analysis

All the experiments were conducted with a minimum of 10 replicates per treatment and repeated 3 times. The data was analysed statistically using SPSS ver 22. The results are expressed as the means ± SEM of three experiments.

Results

Antimicrobial activity

Ethanol and methanol extracts of wild plant leaf and tissue cultured plant samples were analyzed for its antimicrobial activity by agar disc diffusion method. The inhibition zone was measured in mm. Among the four bacterial strains, the highest zone of inhibition was observed in *E. coli* (14±0.82 and 0.8±1.05) at 60 µL concentration of wild and tissue cultured *Cadaba fruticosa* ethanol extracts respectively. The inhibition was observed in a dose depended manner (Table 1).

All the test bacteria showed the highest inhibition zone at 60 µL concentration in wild and tissue cultured plant ethanol extracts whereas in methanol extract the highest zone of inhibition (14±0.14 and 0.9±0.52) was observed in 60 µL concentration against *Streptococcus pyogenes* and *Staphylococcus aureus* respectively (Table 1; Figure 2, 3). The methanol extract of wild *C. fruticosa* reported significant antimicrobial activity against *S. aureus*, *E. coli*, *K. pneumonia* with the zone of inhibition of 12±0.82, 11±0.29 and 11±0.05 respectively (Table 2; Figure 4). Tissue cultured plant methanol extract showed antibacterial potential against *S. aureus*, *E. coli* and *K. pneumonia* with the inhibition zone of 8±0.39, 7±0.71 and 6±0.06 mm respectively (Table 2; Figure 5).

Table 1. Antimicrobial activity of wild and micropropagated plants ethanol extract of *Cadaba fruticosa*

Pathogenic microorganisms	Ethanol extract Zone of inhibition (mm)						Standard (Streptocycline)
	Wild plant ethanol extract			Tissue cultured plant ethanol extract			
	20 µl	40 µl	60 µl	20 µl	40 µl	60 µl	
<i>Streptococcus pyogenes</i>	08±0.12	09±0.72	09±1.05	05±0.08	07±0.91	08±0.04	10±0.33
<i>Staphylococcus aureus</i>	11±0.05	12±0.17	12±0.56	05±0.74	06±0.84	08±0.21	10±0.62
<i>Escherichia coli</i>	10±0.06	12±0.09	14±0.82	05±0.63	06±0.37	08±1.05	13±0.94
<i>Klebsiella pneumoniae</i>	10±1.04	12±0.25	13±0.06	04±0.51	05±0.62	06±0.53	11±0.71
<i>Candida albicans</i>	7±0.93	08±0.71	10±0.32	03±0.39	04±0.08	06±0.72	10±0.52
<i>Trichoderma viride</i>	4±0.37	10±0.37	10±0.83	03±0.28	04±0.11	06±0.31	09±0.71

Table 2. Antimicrobial activity of wild and micropropagated plants methanol extract of *Cadaba fruticosa*

Pathogenic microorganisms	Methanol extract Zone of inhibition (mm)						Standard (Streptocycline)
	Wild plant methanol extract			Tissue cultured plant methanol extract			
	20 µl	40 µl	60 µl	20 µl	40 µl	60 µl	10 µl
<i>Streptococcus pyogenes</i>	10±1.04	10±0.31	14±0.14	04±0.04	06±2.01	08±0.52	10±0.26
<i>Staphylococcus aureus</i>	10±0.83	12±0.09	12±0.82	05±0.32	05±0.06	09±0.39	10±0.41
<i>Escherichia coli</i>	10±0.22	11±0.04	11±0.29	04±0.74	05±0.28	07±0.71	13±0.73
<i>Klebsiella pneumoniae</i>	08±0.51	09±0.37	11±0.05	05±0.61	05±0.91	06±0.06	11±0.25
<i>Candida albicans</i>	10±0.75	07±0.02	09±0.11	03±0.31	05±0.06	06±0.83	10±0.62
<i>Trichoderma viride</i>	03±0.31	08±0.22	10±0.94	03±0.54	04±0.28	05±0.47	09±0.87

Figure 4. Antimicrobial activity of WCFM extract of *Cadaba fruticosa*. **Bacteria:** A- *Escherichia coli*, B- *Klebsiella pneumoniae*, C-*Staphylococcus aureus* and D-*Streptococcus pyogenes*. **Fungi:** E- *Candida albicans* and F- *Trichoderma viride*.

Cytotoxicity study

The higher inhibition percentage in wild and tissue cultured plant ethanol extracts were 53.14 and 54.78 at 5 μ g/ml concentrations (Figure 6). Whereas wild and tissue cultured methanol extracts treatment were 52.58 and 51.39 respectively (Figure 6). The percentage of inhibition increased with the increasing concentration. The IC_{50} value of wild and tissue cultured plant ethanol extracts were 260.36 mg/ml and 268.05 μ g/ml, methanol extract was 273.71 mg/ml and 286.73 μ g/ml (Figure 7) respectively.

Figure 5. Antimicrobial activity of MCFM extract of *Cadaba fruticosa*. **Bacteria:** A- *Escherichia coli*, B- *Klebsiella pneumoniae*, C-*Staphylococcus aureus* and D-*Streptococcus pyogenes*. **Fungi:** E- *Candida albicans* and F- *Trichoderma viride*.

Discussion

Testing of the extracts obtained from wild and tissue cultured plants of *C. fruticosa* for antimicrobial activity showed varying degrees of performance against various microorganisms. The activity against these pathogen might be due to phytochemical constituents of *C. fruticosa*. Mostly pharmacological activity of medicinal plants resides in the so called secondary metabolites, since they are comparatively smaller molecules in contrast to the primary metabolites

Figure 6. Anticancer (HeLa cell line) activity of wild and tissue cultured plant ethanol and methanol extracts of *Cadaba fruticosa*

Figure 7. Inhibition Concentration of Fifty percentage (IC_{50}) of cell viability of HeLa cancer cell line of wild and tissue cultured plant ethanol and methanol extracts of *Cadaba fruticosa*

(Krishnakumar et al., 1997). In the present study, it is observed that the growth of bacterial colonies has been arrested or controlled by the extracts used at different concentrations. Wild and tissue cultured plant ethanol extracts showed higher inhibition zone (14 ± 0.82 and 8 ± 1.05 mm respectively) against *E. coli* at 60 μ l concentration. Similar activity of plant extracts was also observed in *Salmonella typhi* and *Staphylococcus aureus* (Udhaya lavinya et al., 2014). However the wild and tissue cultured plant methanol extracts showed higher antibacterial activity against *Staphylococcus aureus*. The antibacterial potential varied from species to species and also the test organisms (Kalimuthu et al., 2016b). In our study, efficacy of plant extract was directly proportional to its concentration, lower the level of its dilution higher the effective zone of inhibition. This observation in agreement with the findings of Udhaya lavinya et al., (2014) in *Cadaba fruticosa*. Where as in fungal strains, the effect of extracts was less when compared to bacterial strain. This is also in agreement with the earlier reports (Kalimuthu et al., 2016a; Kalimuthu et al., 2016b).

Radiotherapy or surgery is effective when cancer is detected early but many types of cancer are diagnosed when cells from a primary tumor have already metastasized to other parts of the body. At this point the main form of treatment is chemotherapy (Cordero et al., 2012). But most of these drugs cause several side effects in patients. Denny and Wansbrough (1995) reported that a major challenge is to design new drugs that will be more selective for cancer cells and thus have lesser side effects. Combining conventional western medicine with alternative or complementary treatment like herbal medicine, massages Yoga and stress reduction techniques are being used to complement orthodox medicine and treatment approaches in the management of cancer patients (Kam, 2009). According to a 2012 meta-analysis in integrative cancer therapies, more than half of the cancer patients use some kind of integrative therapy (Abidemi et al., 2015).

Plants have been used medicinally for many centuries and are the basics for many synthetic drugs (Marchetti et al., 2012). Around 53% of chemotherapeutic drugs are derived from natural products (Newman and Crass, 2012). The interest in alternative therapies using plant based natural products is increasing Worldwide due to the increase in the number of cancer cases (Jemal et al., 2009). Through *in vitro* and *in vivo* cancer models many plant extracts and its active principles have been studied (Marchetti et al., 2012; Newman and Crass, 2012).

In the present study wild and tissue cultured plant ethanol and methanol extracts showed activity against HeLa cancer cell line with IC_{50} value of 260.36 μ g/ml, 268.05 and 273.71 μ g/ml, 286.75 μ g/ml respectively. Extending literature search showed no report of anticancer activity of *C. fruticosa* against HeLa cancer cell line prior to this study. But (Vadivel et al., 2013). studied the effect of *C. fruticosa* leaf extracts on human lung cancerous cell line A549 and reported that the ethyl acetate extract was found to be more cytotoxicity. However alcohol extracts of leaf exhibited cytotoxicity against Vero, Rhabdo myosarcoma and Hepg-2 cell lines (Mythreyi et al., 2009).

Conclusion

The natural and tissue culture plants of *Cadaba fruticosa* is a potential source of natural antimicrobial and anticancer activity. More or less similar activity was observed in wild and tissue cultured plant extracts. Results of this study are important because these results confirmed the use of tissue cultured plants instead of natural plants. However more investigation are needed to identify the active principles and its molecular mechanism to explain their therapeutic efficacy.

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