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Comparative *In vitro* Cytotoxicity Evaluation of Moringa Extract, Clitoria Extract and *Coccinia grandis* by MTT Assay Using Various Cell Lines

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Authors' contributions

This work was carried out in collaboration among all authors. Author MC designed the study, performed the study, wrote the protocol and wrote the first draft of the manuscript. Authors HT and SS managed the analyses of the study. Author KS managed the final draft of manuscript and additionally did literature searches. Author DG provided the facility for conducting study. All authors read and approved the final manuscript.

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ABSTRACT

Evaluation and development of potent anti-cancer treatments have been a global interest of research due to significant rise in cancer as well as related diseases. Many herbal extracts have shown the potential to be used in the treatment of such diseases. In order to evaluate the anticancer features of *Moringa oleifera* (ME), *Clitoria ternatea* (CE), and *Coccinia grandis* (CCD), this study was carried out using various cell lines i.e. L-929, Vero, L-132, Raw 264.7, and Balb 3T3 with six different concentrations of each test substance by using MTT cell viability Assay. Additionally, *in-vitro* studies are performed using these test items and claims are being done that

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these test items are playing a pivotal role either treatment of Alzheimer's diseases (AD) and Parkinson's disease (PD) or decreasing the severity and incidences of such neurodegenerative diseases. Globally, an average life expectancy rises, so does the severity of these conditions as well. Hence, this study was also aimed to determine whether any supportive changes are showed on different cell lines or not. In contrast to normal cells, the test substances revealed possible anti-cancer characteristics when exposed to carcinoma cell lines based on the data obtained in this study. The results indicated that ME, CE and CCD are effective against cancer cells; hence based on the results of these study, it could be concluded that that the data of the present study supports the interpretation that these test items have significant effects either in treatment or slowing down the clinical signs of PD and AD under the conditions and procedures followed in the present study. Based on the results of cell viability results it could also be concluded that the test items are safer to use up to the concentrations of 500 µg/mL.

Keywords: Cytotoxicity; Moringa oleifera; Clitoria ternatea; Coccinia grandis; anti-cancer; anti-oxidant.

1. INTRODUCTION

The drumstick tree also known as *Moringa oleifera* (ME). It has been suggested by some morphological studies that moringa extract may possess anti-cancer properties, as evidenced by the reduction of cell size, blebbing of cells, the condensation of chromatin, and the nuclear fragmentation of cells. Bhadresha et al., [1]. It has been demonstrated that an extract of *Moringa oleifera* leaves can halt the spread of cancer cells [2]. One of the most common diseases affecting people is cancer, which is often incurable [3]. It is estimated that there would be 585,720 cancer deaths and 1,665,540 new cancer cases in the US in 2014 Siegel R et al., [4]. A normal cell undergoes apoptotic cell death, which is a self-destructive metabolism in accordance with the genetically programmed cell death signal, when its DNA or other components are externally damaged by different sources [5]. However, the primary significance of anticancer medicines lies in their ability to heal a compromised system. Anticancer drugs work by disrupting the cancer cells' ability to proliferate while inducing the apoptosis signalling pathway [6]. However, their nonspecific activities and toxicity to normal cells sometimes restrict the number of medications accessible for the care of malignancies [7]. Flavanoid glycosides have been reported to be present in the flowers of *Clitoria ternatea* [8,9]. Ancient medicine from Ayurveda *Clitoria ternatea* (CE), often known as "Butterfly pea," has been utilized as an anti-stress, anxiolytic, antidepressant, and anticonvulsant drug. In addition, a variety of pharmacological properties, including as antibacterial, antipyretic, and anti-inflammatory properties, are present in its extracts [10] The fruits of the ivy gourd *Coccinia grandis* (CCD) have been used to cure leprosy, fever, asthma, bronchitis, and jaundice [11,12]. All three of these test items are also utilized in Parkinson's and Alzheimer's disease, both of which are progressive brain conditions brought on by slow cell death in the brain [13]. All three herbs are used commonly as herbal preventative medicine or for the treatment of certain diseases. Therefore, it is important that the toxicity or safety of these plants/herbs be assessed. Therefore, the goal of the current study is to assess in vitro cytotoxicity using MTT Assay (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide), which will provide first, preliminary data that may be helpful for future investigations on the safety of these plants. This study aims to determine if *Moringa oleifera*, *Clitoria ternatea*, and *Coccinia grandis* have any possible anti-cancer, Parkinson's and Alzheimer's disease treatment-related benefits on L-929, Vero, and, L-132, Raw 264.7, and Balb 3T3 Cell Lines.

2. MATERIALS AND METHODS

2.1 Materials

Cell culture media; Minimum Essential Medium (MEM), Dulbecco's Modified Eagle's Medium (DMEM), Fetal bovine serum (FBS), Cell culture plates (96 well plates), trypan blue dye, consumables and trypsin-EDTA (0.25%) were procured from Himedia, Maharashtra, India. MTT and other specified chemicals and solvents were obtained from Sigma. Cyclophosphamide Monohydrate (positive control) was procured from Tokyo Chemical Industry Co. Ltd., High-Density Polyethylene (HDPE) beads (Negative control) were procured from Good Fellow Cambridge Limited.

2.2 Cell Line Maintenance

Details of Cell lines used for this research are mentioned in Table 1. All Cell lines were procured from National Centre for Cell Science (NCCS), Pune, India.

2.3 Sample Preparation

Cytotoxicity test was performed for *Moringa oleifera*, *Clitoria ternatea* and *Coccinia grandis* using different cell lines viz. L-132, L-929, Vero, Raw 264.7 and Balb 3T3. Solubility and precipitation check for all three test items were performed in culture media supplemented with 10% FBS and Antibiotic-Antimycotic Solution [14,15]. All three Test items were found soluble in culture media and the highest treatment concentration selected for further experiment was 1000 µg/mL. After preparing stock concentration of each test item, six concentrations (1000 µg/mL, 500 µg/mL, 250 µg/mL, 125 µg/mL, 62.5 µg/mL, and 31.25 µg/mL) were prepared from stock by serial dilution. Complete cell culture media was used as vehicle control. Negative control extraction was prepared using 0.2 g/mL of extraction criteria. Extractions were prepared followed by incubation at 37 °C for 24 h. Positive Control was freshly prepared by diluting cyclophosphamide monohydrate in culture media at concentration 10 mg/mL and used.

2.4 Experimental Design (MTT ASSAY)

Cytotoxicity testing using cell cultures is a quick, systematic, accurate, and affordable method of determining if a substance contains significant quantities of biologically harmful extractable. The cytotoxicity study of *Moringa oleifera*, *Clitoria ternatea* and *Coccinia grandis* extract was studied on cultured cells using MTT Assay [16]. L-132, L-929, Vero, Raw 264.7, and Balb 3T3 cell lines were exposed to each of the three test items. These five Cell Lines were seeded in 96 well plates. The cells were grown in 96 well plates for the establishment of monolayer till 80% confluences after an incubation period for 24 hours in CO_2 incubator at 5 % CO_2, 37 °C, ≥90 % relative humidity. 100 µL of vehicle control, negative control, positive control, and test item concentrations (1000 g/mL, 500 g/mL, 250 g/mL, 125 g/mL, 62.5 g/mL, and 31.25 g/mL) were added to the respective wells in triplicates after the incubation period was complete. Cells were incubated further in to CO_2 incubator for 24 hours, at 5 % CO_2, 37 °C, ≥90 % relative humidity. After duration of exposure, microscopic examination was done to assign the reactivity grades inspired from ISO 10993-5:2009 guideline [17]. The culture media was taken out of every well on the plates after it had been examined. 50 µL of MTT solution (1 mg/mL) was added to each test well of culture plates and incubated further for 2 h in CO_2 incubator. The MTT solution was decanted carefully and 100 µL of isopropanol was added in each well followed by incubation of 10 minutes at room temperature to dissolve the formazan crystals completely. The colour of each well was observed visually [18]. Ninety-six well plates were transferred into a microplate reader equipped with a 570 nm to read the absorbance.

Table 1. Details of cell lines

Name of Cell line	Origin	Tissue	Complete Growth Medium	Culture Conditions
L-929	Mus musculus, Mouse	Connective Tissue	Minimum Essential Medium (MEM), with 10% Fetal Bovine Serum (FBS), Sodium bicarbonate, 100 IU/mL Penicillin, 100 µg/ml streptomycin.	Temperature: 37 ± 1 °C, Carbon dioxide (CO_2): 5 ± 0.5 % and Relative Humidity ≥ 90%.
Vero	African green monkeys	Kidney		
L-132	Human	Embryonic lung		
Raw 264.7	Mouse	Abelson murine leukemia virus - induced tumor	Dulbecco's Modified Eagle's Medium (DMEM), with 10% Fetal Bovine Serum (FBS), Sodium bicarbonate, 100 IU/mL Penicillin, 100 µg/ml streptomycin.	
Balb-3T3	Mouse	embryo		

3. RESULTS AND DISCUSSION

3.1 Qualitative Assay (Microscopic Examination)

In vitro cytotoxicity testing of three test items was performed using L-929, L-132, Vero, Balb 3T3 and Raw 264.7 cell lines following the procedure inspired from ISO 10993-5:2009 guideline. Three test items were evaluated for cytotoxicity using six concentrations (1000 µg/mL, 500 µg/mL, 250 µg /mL, 125 µg /mL, 62.5 µg /mL, 31.25 µg /mL). At the concentration of 500 µg/mL, *Coccinia grandis* was found mild reactive (Grade: 2) to L-929 cell line and slightly reactive (Grade: 1) to Vero cell line. At the concentration of 500 µg/mL, *Clitoria ternatea* was found mild reactive (Grade: 2) to L-929 cell line and slightly reactive (Grade: 1) to Vero cell line. At the concentration 500 µg/mL, *Moringa oleifera* was found slightly reactive (Grade: 1) to L-929 cell line whereas no reactivity (Grade: 0) was found in Vero cell line. All the lower concentrations (250 µg /mL, 125 µg /mL, 62.5 µg /mL, 31.25 µg /mL) had shown no reactivity (Grade: 0) for L929 and Vero cell lines.

The highest concentration 1000 µg/mL, *Coccinia grandis* was found Severe reactive (Grade: 4) to Balb 3T3 and L-132 cell lines whereas mild reactive (Grade: 2) to Raw 264.7 cell line. Lower concentration 500 µg/mL was found slightly reactive (Grade: 1) to Balb 3T3 and Raw 264.7 cell lines whereas mild reactive (Grade: 2) to L-132 cell line. All the other lower concentrations (250 µg /mL, 125 µg /mL, 62.5 µg /mL) had shown no reactivity (Grade: 0). the highest concentration 1000 µg/mL, *Clitoria ternatea* was found severe reactive (Grade: 4) to Balb 3T3, L-132 and Raw 264.7 cell lines. The lower concentrations (500 µg/mL and 250 µg /mL) were also found severe reactive and 125 µg /mL was found mild reactive to Balb 3T3 cell line. The concentrations (500 µg/mL, 250 µg /mL and 125 µg /mL) were found severe reactive and 62.5 µg /mL was found mild reactive to L-132 cell line. Concentrations (500 µg/mL and 250 µg /mL) were found mild and slight reactive respectively whereas 125 µg /mL had shown no reactivity (Grade: 0). The highest concentration 1000 µg/mL, *Moringa oleifera* was found severe reactive (Grade: 4) to Balb 3T3 cell line whereas mild reactive (Grade: 2) to L-132 and Raw 264.7 cell lines. The lower concentration (500 µg/mL) had shown mild reactivity to Balb 3T3 and L-132 cell lines whereas slight reactive to Raw 264.7 cell line. The lower concentration (250 µg /mL) was slight reactive to L-132 cell line. Other concentrations (250 µg /mL, 125 µg /mL, 62.5 µg /mL) had shown no reactivity (Grade: 0) to all three cell lines.

3.2 Quantitative Assay

Cell viability for the different dilution of test item and related cytotoxicity grades were as below:
Calculate % cell viability using following equation:

> % Cell Viability= OD of Treated / OD of Control ×100

Where,

> **OD of Treated:** The mean value of the measured optical density of the concentrations of the test item or Positive or Negative control;
>
> **OD of Control:** The mean value of the measured optical density of the vehicle control.

At the concentration 500 µg/mL, *Coccinia grandis* Cell viability percentage was estimated 83% and 97% for L-929 and Vero cell lines respectively. At the concentration 500 µg/mL, *Clitoria ternatea* cell viability percentage was estimated 84% and 74% for L-929 and Vero cell lines respectively. At the concentration 500 µg/mL, *Moringa oleifera* cell viability percentage was estimated 87% and 86% for L-929 and Vero cell lines respectively. Cell viability percentage were obtained greater than the acceptable limits of 70% for all the lower concentrations (250 µg /mL, 125 µg /mL, 62.5 µg /mL, 32.25 µg /mL) for L929 and Vero cell lines.

The highest concentration 1000 µg/mL, *Coccinia grandis* Cell viability percentage was estimated 54%, 52% and 77% for Balb 3T3, L-132 and Raw 264.7 cell lines respectively. Cell viability percentage were obtained greater than the acceptable limits of 70% for all the lower concentrations (500 µg/mL, 250 µg /mL, 125 µg /mL, 62.5 µg /mL). The highest concentration 1000 µg/mL, *Clitoria ternatea* cell viability percentage was estimated 42%, 35% and 40% for Balb 3T3, L-132 and Raw 264.7 cell lines respectively during MTT based cell viability assay. The highest concentration 1000 µg/mL, *Moringa oleifera* cell viability percentage was estimated 47%, 51% and 71% for Balb 3T3, L-132 and Raw 264.7 cell lines respectively during MTT based cell viability assay. For *Clitoria ternatea* and *Moringa oleifera* cell viability

percentage was obtained greater than the acceptable limits of 70% for the lower concentrations (250 µg /mL, 125 µg /mL, 62.5 µg /mL) for Balb 3T3, L-132 and Raw 264.7 cell lines. Summary of the results is mention in Table 2.

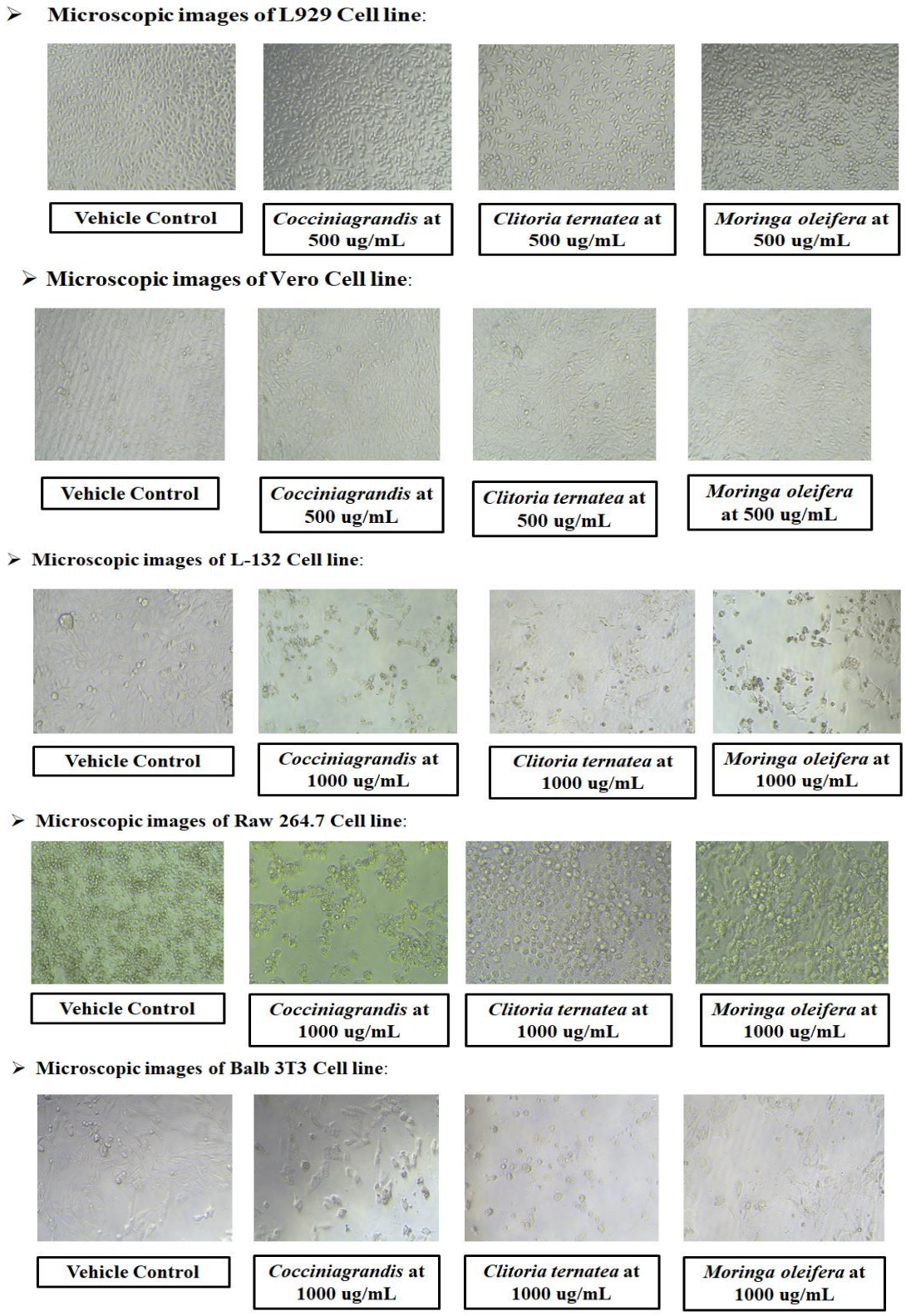

Fig. 1. Microscopic images of different cell lines

Table 2. Summary of the results

Test Item Conc. (µg/mL)	Test Item Concentrations vs Cell lines Viability														
	L-929			Vero			Balb 3T3			Raw 264.7			L-132		
	CCD	CE	ME	CCD	CE	ME	CCD	CE	ME	CCD	CE	ME	CCD	CE	ME
1000	-	-	-	-	-	-	54	42	47	77	40	71	52	35	51
500	83	84	87	97	74	86	77	51	78	91	76	81	87	41	71
250	114	109	96	95	88	87	86	60	97	100	84	95	100	47	88
125	120	110	97	92	89	86	92	76	95	109	87	99	105	58	92
62.5	118	118	101	93	90	84	92	90	101	112	94	110	106	79	101
31.25	125	118	104	92	91	85	-	-	-	-	-	-	-	-	-

**Keys: Clitoria ternatea (CE), Coccinia grandis (CCD) and Moringa oleifera (ME)*

4. CONCLUSION

Based on qualitative observations and quantitative evaluation in this study, test item '*Coccinia grandis*' was found reactive at concentration of 1000 µg/mL to Balb 3T3, Raw 264.7 and L-132 cell lines. Test item '*Clitoria ternatea*' was found reactive at the concentrations of 1000 µg/mL, 500 µg/mL, 250 µg /ml and at 125 µg /mL to L-132 cell line. Additionally, at the concentration of 1000 µg/mL Raw 264.7 cell line found cytotoxic. Test substance '*Moringa oleifera*' was found reactive at the highest concentration (1000 µg/mL) to Balb 3T3, Raw 264.7 and L-132 cell lines. It can be concluded that all the three test substances are safe to use at lower concentrations (500 µg/mL, 250 µg /mL, 125 µg /mL, 62.5 µg /mL, 31.25 µg /mL) for the treatment of diseases like Parkinson's, Alzheimer's and neurodegenerative disorders. All three test items are showing cytotoxicity against cancerous cell lines L-132 and Balb 3T3 at 1000 µg/mL concentrations; based on this effect it could be concluded that the *in vitro* cytotoxicity test revealed that these test items has shown activity against Cancerous cell lines; which might be a signal towards their anticancer activities. This effect could be confirmed by further testing of these test items.

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COMPETING INTERESTS

Authors have declared that they have no known competing financial interests or non-financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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