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IN VITRO EVALUATION OF ETHANOLIC EXTRACT OF FRESHLY PROCESSED (VIRALIMANJAL VARIETY) *Curcuma longa* FOR THE ANTIMICROBIAL, ANTIOXIDANT AND CYTOTOXIC PROPERTIES

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ABSTRACT

Curcuma longa, popularly known as turmeric is a spice used for cooking for centuries in India which is known for its antimicrobial, anticancer, antioxidant, anti-inflammatory properties etc. The recent rise in cases of antimicrobial resistance to conventional medicines has prompted mankind to discover alternative medicines for treatment and alleviation of cancer and infectious diseases. Medicinal plants have long been used for several millennia for treatment and alleviation of diseases. One such medicinal plant which is used in several traditional systems of medicine is *Curcuma longa*, popularly known as turmeric. Antioxidant activity is essential because oxidative stress is linked to non-communicable diseases such as cancer, diabetes and inflammation. This paper has investigated the antimicrobial, antioxidant and cytotoxic properties of the ethanolic extract of *Curcuma longa*.

KEYWORDS: Antioxidant, *In vitro*, *Curcuma longa*, antimicrobial, toxicity.

INTRODUCTION

Reactive Oxygen species and Reactive Nitrogen species are generated constantly in the living body and are over-produced in pathological conditions resulting in oxidative stress therewith. To protect their molecular systems, all living oxygen - consuming organisms are endowed with an antioxidant system including enzymatic and non-enzymatic components. Antioxidants can eliminate those which can induce free radical stress. The most important sources of antioxidants are mainly medicinal plants (Karadeniz et al., 2015). Many studies have been conducted on medicinal plants with respect to antioxidant studies. Guo et al have determined the antioxidant activity of peel, pulp and seed fractions of fruits commonly consumed in China (Guo et al., 2003).

Balashanmugam P., MosaChristas and Kowsalya have determined the in vitro cytotoxicity and antioxidant activity of gold nanoparticles green synthesized using *Marselia quadrifolia* leaf extract. They have characterized the nanoparticles using HR-SEM, EDAX, XRD and UV-Visible (High-Resolution Scanning electron microscopy, Energy dispersion X-Ray, X-Ray diffraction) spectrometry techniques. Nanotechnology and nanomaterials have also gained attention in recent times due to their surface-volume ratio, and their excellent efficacy in treatment of diseases. Nanotechnology has also gained an important role in medical imaging (Balashanmugham P. et al., 2018).

Sarfaraz et al have determined the essential oil composition and antioxidant activity of oregano and marjoram as affected by light emitting diodes. In *O. marjorana*, the blue-red treatment led to the increase in production of the essential oil. (Sarfaraz et al., 2023)

Nimal Christhudas et al have evaluated the antidiabetic, antioxidant potential including free radical scavenging activities of *Hedyotis biflora*. Appreciable antidiabetic, antioxidant along with free radical scavenging abilities were determined. This study proved that antioxidant ability has a clear link in mitigating the problems and complications arising out of diabetes mellitus (DM) (Nimal Christhudas et al., 2013). This was indicated by higher scavenging of DPPH and nitric oxide free radicals apart from higher inhibition of alpha-glucosidase, the marker responsible for diabetes mellitus (DM).

Galib and Algfri have determined the free radical scavenging capability of DPPH of leaf extract of *Capparis cartilagenia*. In this paper, they have used DPPH scavenging in bioautography using a TLC Plate. The formation of yellow spots determined the antioxidant activity i.e., free radical scavenging activity of the plant extract mentioned. (Galib & Algfri, n.d.).

Lidiane Diniz do Nascimiento et al have described and reviewed the bioactive properties of various spices commonly used in Brazil namely cinnamon, peppermint, oregano, thyme, basil and rosemary. These spices are being used for several centuries and they possess remarkable antimicrobial and antioxidant properties (Diniz Do Nascimento et al., 2020).

OBJECTIVE

The objective of the present study is to develop a suitable drug using processed *Curcuma longa* rhizome powder for antimicrobial, antioxidant evaluations *in vitro*.

MATERIALS AND METHODS

Ethanolic Extract Preparation: 5 g of freshly processed ‘’viralimanjal’’ variety of powdered *Curcuma longa* rhizomes were dissolved in 75 mL of ethanol. The extract was then obtained by evaporating in rotary vacuum evaporator until an orangish brown liquid was obtained.

Phytochemical Analysis: The Phytochemical analysis was carried out for detecting the qualitative presence of alkaloids, phenolics, flavonoids, tannins and proteins as per a modified protocol of Shaikh and Patil (Shaikh & Patil, 2020).

1. **Detection of alkaloids**: To the extract, 3-4 drops of picric acid solution was added, dark orangish brown precipitate indicates the formation of alkaloids.
2. **Detection of flavonoids**: To the extract, 1 mg/ mL of lead acetate solution was added and formation of greenish-yellow solution indicated formation of flavonoids.
3. **Detection of phenolics**: To the extract, 1 mg/ mL of ferric chloride solution was added. Formation of dark green colour indicated the formation of phenolics.

4. **Detection of tannins**: To the extract, 1 mg/ mL of Ferric chloride solution along with a few drops of water were added simultaneously. The formation of dark green colour indicated the presence of tannins.
5. **Detection of proteins**: To the extract, a few drops of concentrated nitric acid were added. Formation of a change in color indicated the presence of proteins.

Antibacterial activity: The effect of samples against *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa* and *Streptococcus pyogenes* was investigated by the well-diffusion method (Perez, 1990). The pure cultures of bacterial pathogens were obtained and maintained in nutrient agar (NA) slant at 37ºC. The bacterial strain was grown overnight in nutrient broth on a rotary shaker (200 rpm) at 37ºC. The inoculum containing a microbial load of 1×10^5 CFU/ml was then swabbed to the nutrient agar medium plate. Wells of 3 mm diameter were punched aseptically with a sterile cork borer and further loaded with different samples and 20 µg of Gentamycin (positive control for bacteria). The plates was then incubated for 24 hrs at 37ºC and the zone of inhibition (ZOI; mm) appearing around the wells was measured. (Perez et al 1990). The activity was evaluated by Dr. P. Balashanmugham at Avigen Biotech, Chromepet, Chengalpattu District, TN, India.

DPPH scavenging activity: The DPPH scavenging activity of the sample was measured by 1,1-diphenyl-2-picrylhydrazyl (DPPH) method (Manzocco et al., 1998). Briefly, 0.4mM solution of DPPH in methanol was prepared and 2 mL of this solution was added to different concentrations of sample and was allowed to stand at room temperature for 15 mins, and then absorbance was read at 517 nm against blank samples. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The percentage of the DPPH radical scavenging is calculated using the equation as given below:

Radical scavenging activity/Inhibition (%) = (Absorbance control – Absorbance sample / Absorbance control) x 100. The activity was evaluated by Dr. P. Balashanmugham at Avigen Biotech, Chengalpattu District, TN, India.

Cytotoxicity Analysis for In vitro Toxicity:

Protocol:

Cell Culture Maintenance

Vero (African green monkey kidney normal epithelial cell line) was obtained from the National Centre for Cell Sciences (NCCS), Pune, India. Cells were maintained in the logarithmic phase of growth in Dulbecco's modified eagle medium (DMEM) supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin. They were maintained at 37°C with 5% CO_2 in 95% air humidified incubator.

Cytotoxic effect

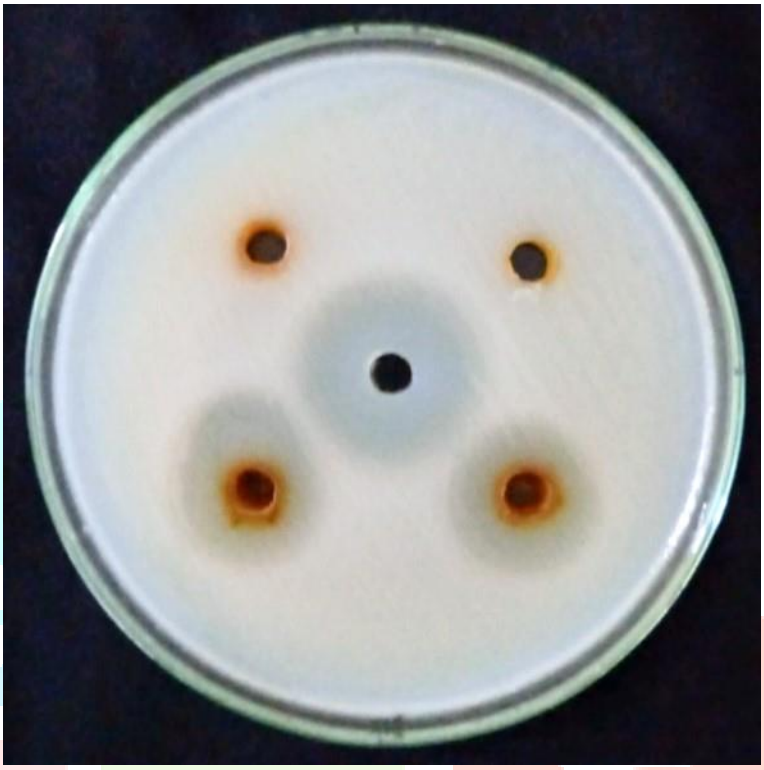
The cytotoxic effect of the sample was tested against Vero cell line by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Mossman, 1983). The cells were seeded in 96-well microplates (1 x 10^6 cells/well) and incubated at 37°C for 48 h in 5% CO_2 incubator and allowed to grow 70-80% confluence. Then the medium was replaced and the cells were treated with different concentrations of sample and incubated for 24 h. The morphological changes of untreated (control) and the treated cells were observed under digital inverted microscope (20X magnification) after 24 h and photographed. The cells were then washed with phosphate-buffer saline (PBS, pH-7.4) and 20 µL of (MTT) solution (5 mg/mL in PBS) was added to each well. The plates were then made to stand at 37ºC in the dark for 2 h. The formazan crystals were dissolved in 100 µL DMSO and the absorbance was read spectrometrically at 570 nm. Percentage of cell viability was calculated using the formula,

Cell viability (%) = (Absorbance of sample/Absorbance of control) X 100. (T. Mossmann 1983).

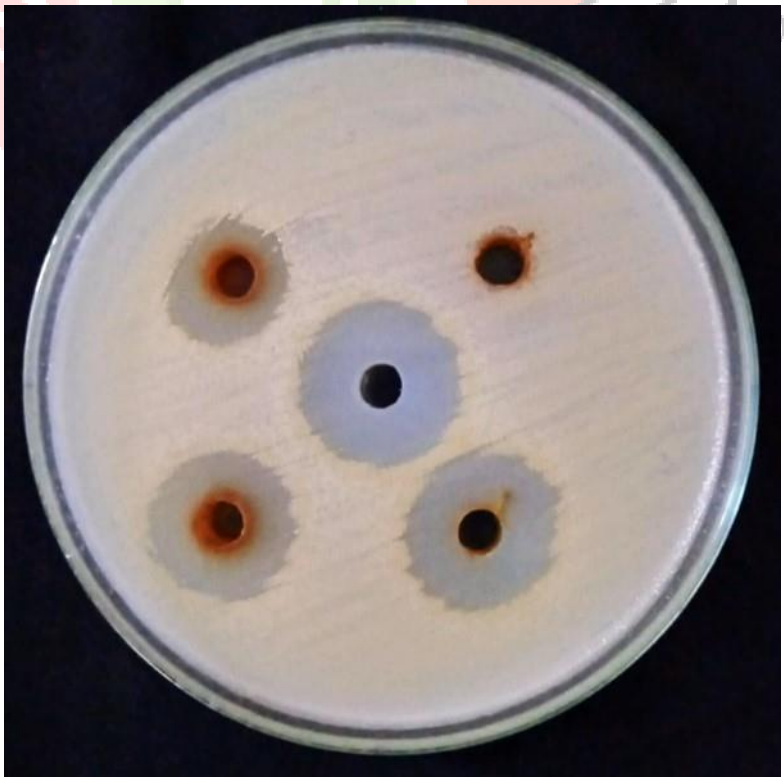
The assay was evaluated by Dr. P. Balashanmugham at Avigen Biotech, Chengalpattu District, TN, India.

RESULTS

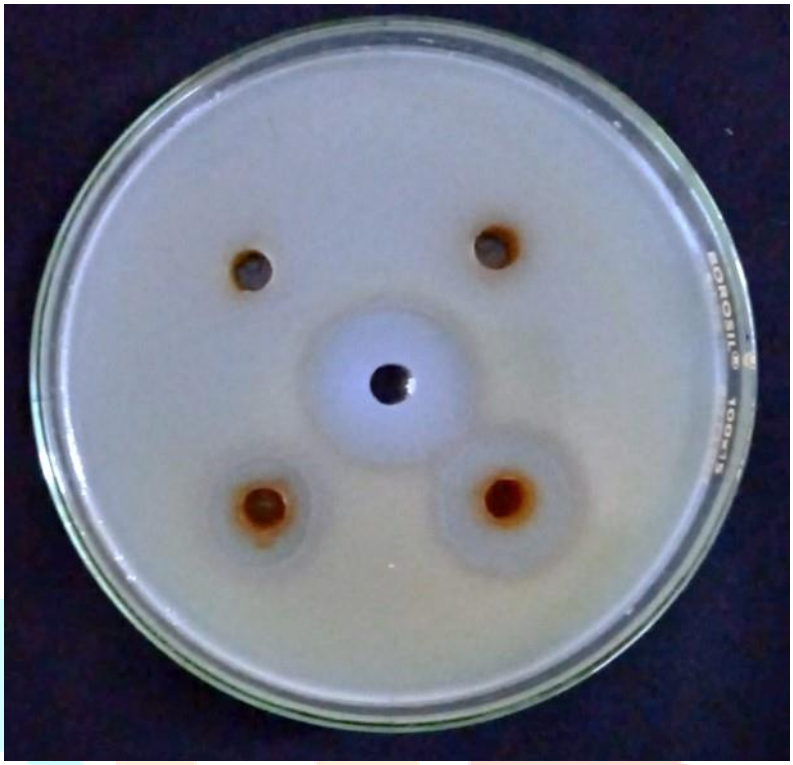
(I) IMAGES FOR THE ANTIBACTERIAL ASSAYS OF *Curcuma longa* ethanolic extract at different concentrations.



(A) Legend: *Bacillus subtilis*

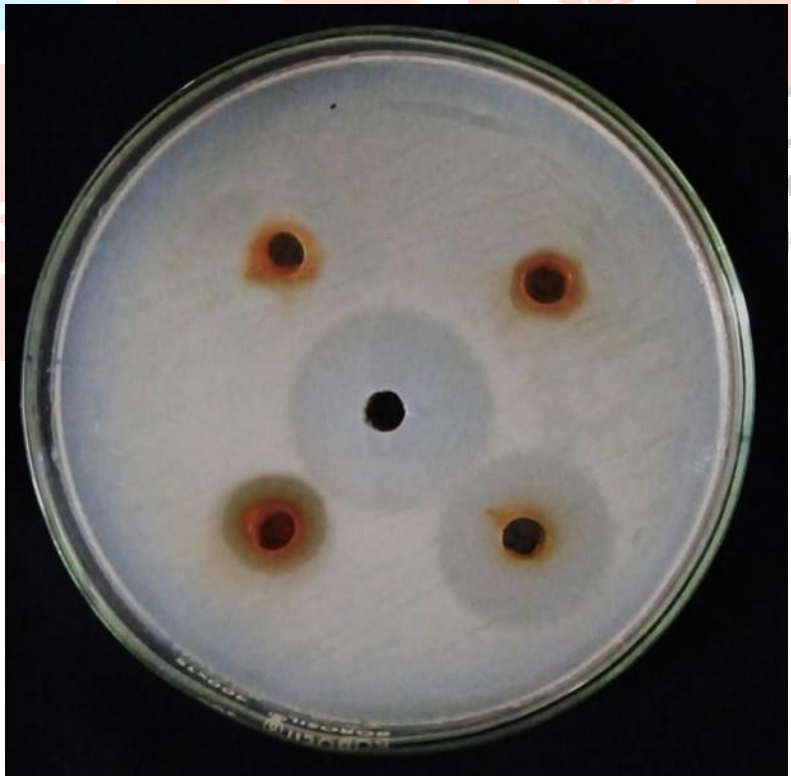


(B) Legend: ***Staphylococcus aureus***



(C)

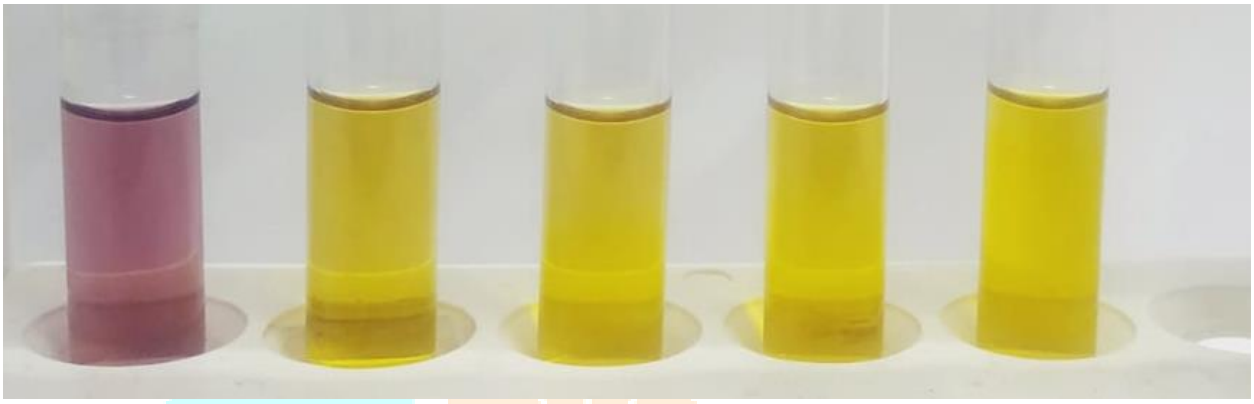
Legend: ***Pseudomonas aeruginosa***



(D) Legend: ***Escherichia coli***

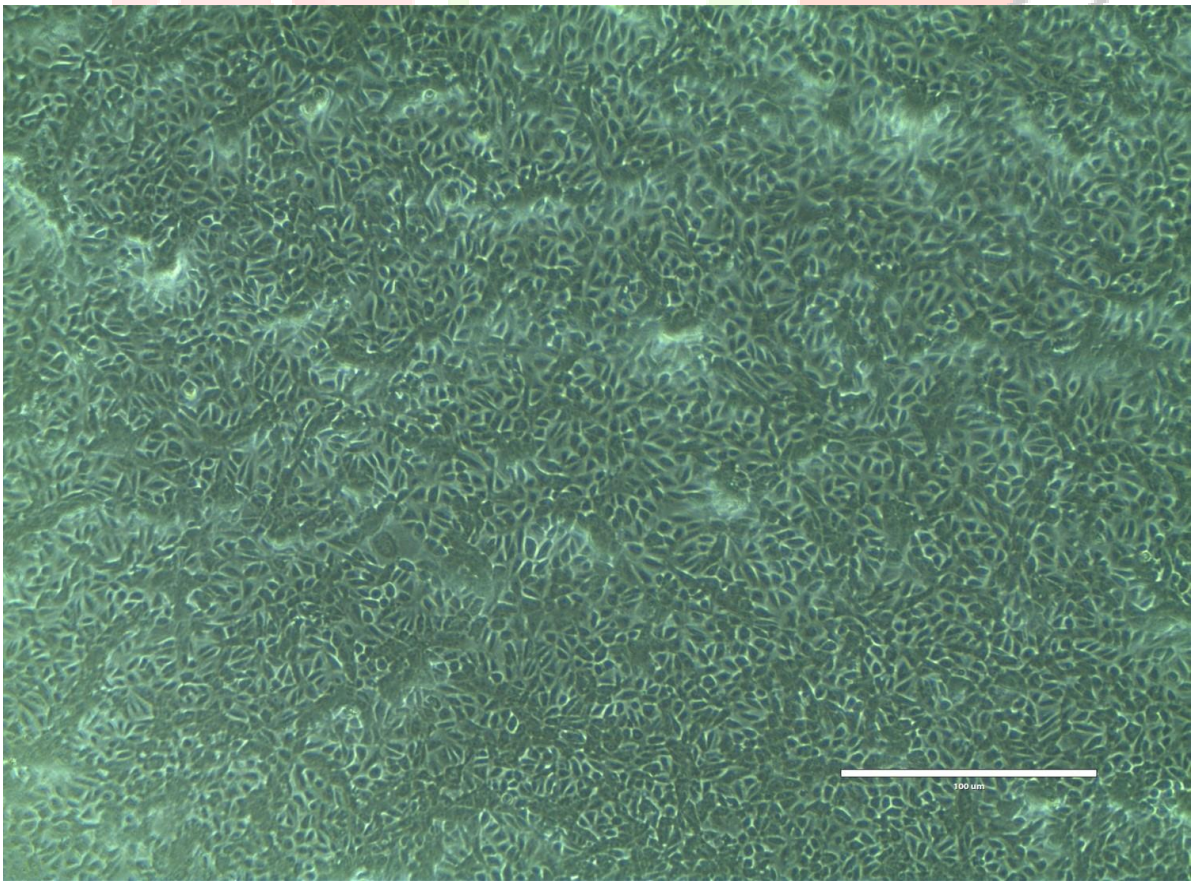
The central zone for each represents the positive control (gentamycin)

(II) IMAGE FOR ANTIOXIDANT ASSAY

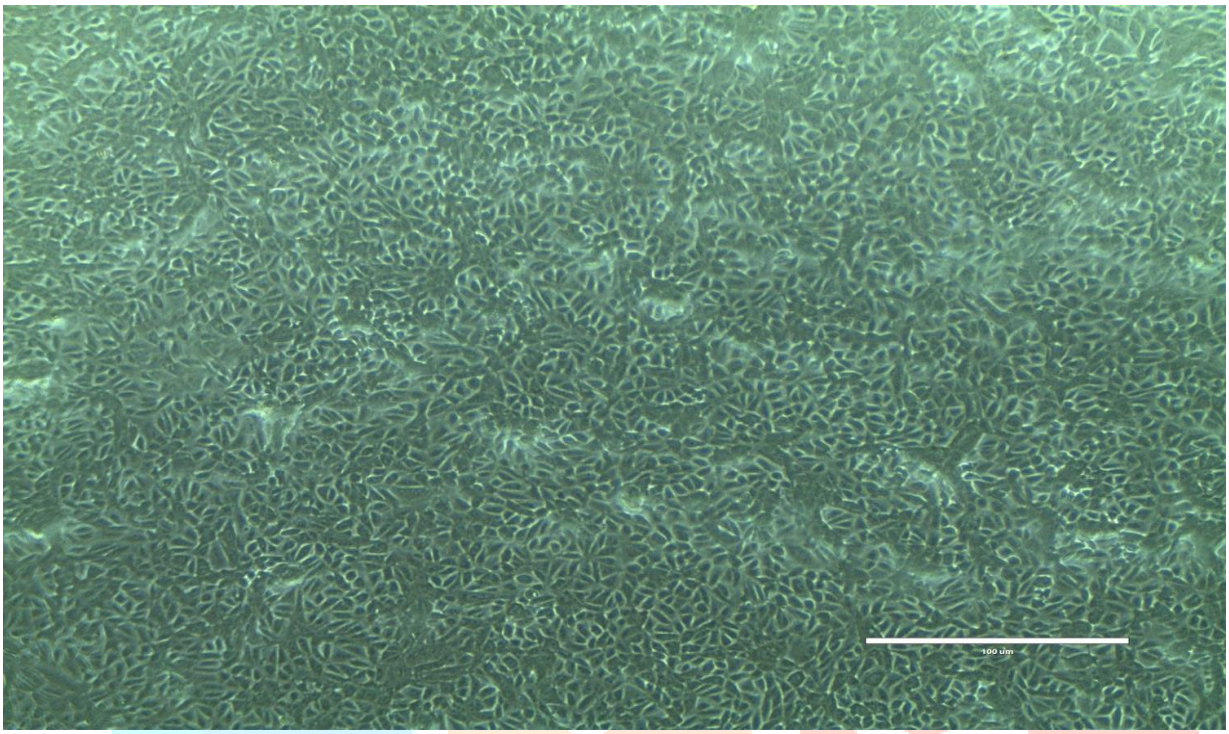


Legend: The Control is DPPH dissolved in Methanol solution (Negative control).

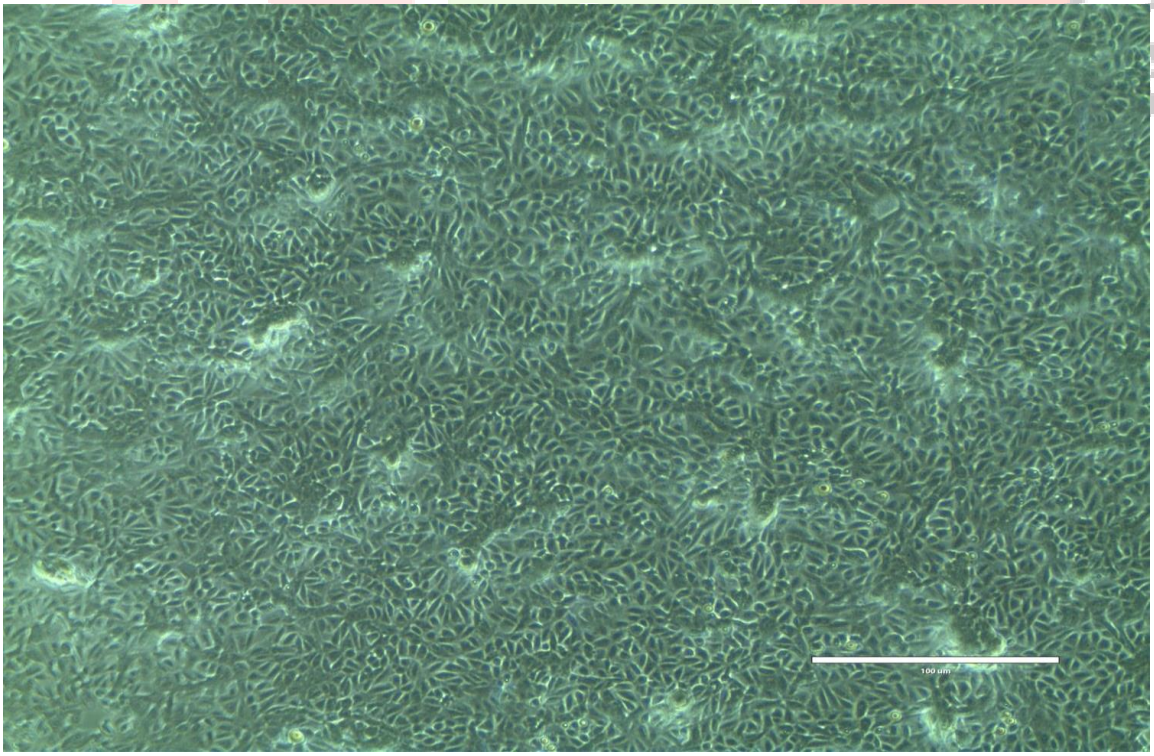
(III) IMAGES OF *IN VITRO* TOXICITY ON VERO CELLS



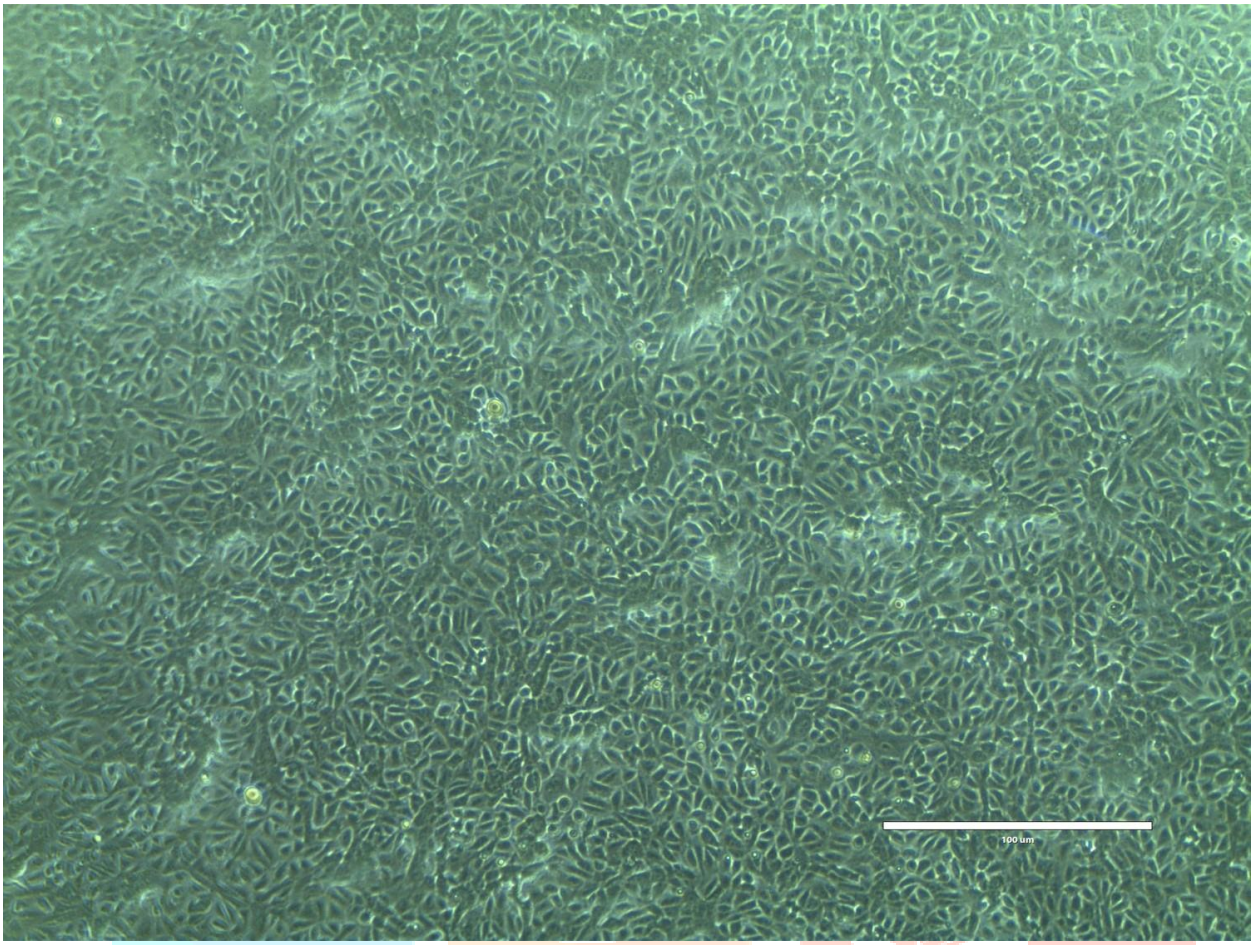
Legend: Vero cells treated at 25 µg/ mL concentration of ethanolic extract of turmeric rhizome powder. Cell viability is seen.



Legend: Vero cells treated at 50 µg/ mL of ethanolic extract of turmeric powder. Cell viability is seen here.



Legend: Extract treated at 75 µg / mL concentration of ethanolic extract of powdered rhizomes of *C. longa*. Cell viability is visible here.



Legend: Cell viability at 100 µg/ mL concentration of ethanolic extract of powdered turmeric rhizomes (*C. longa*). Viability of cells are seen.

TABLES

Table no: 1

Pathogens	Zone of inhibition (mm)				
	25 µg/ml	50 µg/ml	75 µg/ml	100 µg/ml	Positive Control (gentamycin 25 µg)
B. subtilis	0	0	17	19	22
S. aureus	0	16	18	20	22
P. aeruginosa	0	0	14	19	21
E. coli	0	0	12	22	26

Legend: Antibacterial activity Table. *Curcuma longa* ethanolic extract.

Table no:2

Concentrations (µg/ml)	**Absorbance**			**Average**	**Inhibition (%)**
	I	II	III		
Control	0.978	0.986	0.982	0.982	
25	0.529	0.528	0.522	0.526	46.38832
50	0.337	0.331	0.339	0.335	65.81806
75	0.277	0.277	0.279	0.277	71.72437
100	0.218	0.225	0.226	0.223	77.29124

Legend: Antioxidant Activity: DPPH Scavenging Activity Table *Curcuma longa* ethanolic extract.

Table no: 3

Concentrations (µg/mL)	**Absorbance**		**Average**	**Cell Viability (%)**
	I	**II**		
Control	0.751	0.755	0.753	100
25	0.747	0.751	0.749	99.4687915
50	0.743	0.74	0.7415	98.47277556
75	0.729	0.724	0.7265	96.48074369
100	0.718	0.713	0.7155	95.01992032

Legend: In vitro Toxicity on Vero cells. *Curcuma longa* ethanolic extract

Table 4: Phytochemical Analysis of *C. longa* Ethanolic extract.

Phytochemicals	Test Used	Present/Absent
Alkaloids	Picric Acid Test	Positive
Flavonoids	Lead Acetate Test	Positive
Tannins	Tannic Acid Test	Positive
Phenolics	$FeCl_3$ Test	Positive
Proteins	Nitric Acid Test	Positive

DISCUSSION

Curcuma longa is a traditional spice which is used in cooking and in folk medicine since antique times. The antimicrobial, anticancer, antioxidant, anti-inflammatory properties of this spice are well known to mankind. Here, we have evaluated the antimicrobial, antioxidant and the in vitro toxicity of the viralimanjal variety of the processed powder of *C. longa*. Amarasuriyan et al have conducted an ethnobotanical survey of medicinal plants whereby *Curcuma longa* i.e., viralimanjal variety was found to be in use. (Amarasuriyan et al.,2013). Kovacik et al reported an adverse cytotoxic effect of gentamycin on vero cell line at 2000 and 4000 µg/mL (Kovacik et al., 2017). When compared with the report of Kovacik et al, our extract showed extremely less cytotoxicity to Vero cells. Moreover, our extract showed excellent antibacterial activity. The increase in resistance to antibacterials in recent times has forced mankind to look for alternative drugs. Phytochemicals have been an excellent choice here and so; our extract has showed good activity as against all the four pathogens. Also, Good antioxidant activity has been reported by our team by DPPH radical decolorization activity.

Alvarado et al have evaluated the antimicrobial activity of medicinal plants as against bacteria related to equine infections. The bacteria *Escherichia coli, Salmonella enterica, Staphylococcus aureus* and *Listeria monocytogenes* are the common etiological agents responsible for diseases in horses. The two medicinal plants namely *Cuphea aequipetala* and *Eryngium cornosum* were found to exhibit antimicrobial activity at 50 % ethanolic extracts (Alvarado et al 2020). Our results are found to be greater in magnitude than the report of Alvarado et al. This is because in the report of Alvarado et al, the zones of inhibition were in the range of 8-9 mm whereas in our case,

bigger zones of inhibition were recorded in 20-22 mm at 100 µg/ mL concentration. Wang and Zhang (Wang and Zhang 2020) have made a theoretical investigation in to the mechanism of scavenging of DPPH free radical using edaravone. The DPPH scavenging process is a two step process which involves H- atom abstraction process and a proton concerted electron transfer process.

$DPPH_{\circ} + RXH \rightarrow DPPHH + RX_{\circ}$ - (1)

$DPPH_{\circ} + RXH \rightarrow DPPH^{-} + RXH_{\cdot}^{+}$

$\rightarrow DPPHH + RX.$ - (2)

The two equations (1) and (2) are representative of the mechanism of DPPH quenching activity of antioxidants. In our study, it is hypothesized that DPPH is scavenged in a similar way. Our study is an *in vitro* study whereas the report of Wang and Zhang is an *in silico* approach as they have employed molecular docking. The confirmatory test behind DPPH radical scavenging activity is that the purple DPPH radical is converted to a colorless solution. From the images, a yellow solution is formed post DPPH scavenging in our study indicating the presence of phytocompounds and the possibility of pigments in our study.

CONCLUSION

With this, it is inferred that *Curcuma longa* (viralimanjal variety) in processed form through our experiments and findings has showed an excellent antimicrobial and antioxidant activity with very less toxicity.

FUTURE WORK

The chemical characterization and *in vivo* evaluation of the ethanolic extract of *C. longa* is to be carried out in a more extensive and exhaustive study.

ACKNOWLEDGEMENT

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