Screening the Cytotoxic Effect of *Terminalia chebula* by *in vitro* Using L-929 Fibroblast Cell Line

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***Abstract*: Low cost and fewer side effects are major reasons for choosing plant and herbal based products among the populations. WHO also recommended the use of herbal based formulation and most of the developing countries are now utilizing the herbal products for various types of illness. Most herbal products are not safe for use without any scientific validation be causing side effects due to the presence of certain phytochemical compounds. In our study reveals the toxic effect of *Terminalia chebula* by *in-vitro* methods using L929 fibroblasts cell lines. Various concentrations of herbal concentration were not affect the cell viability and the extract had not held any toxic effect.**

***Keywords*: Cytotoxicity, *Terminalia chebula,* Phytochemical, Medicinal plants, Cytotoxic Effect, Fibroblast cell line**

Introduction

Many homelands in India are closely associated with household and industry-based animal propagation. Diary animals like cows are of significant concern for the people in the bottom layer of the system. The economy was also relied on this animal cultivation along with agriculture. It is still practiced by people especially farmers in India and even treats it as a divine process to maintain cows. Thus the healthy maintenance of the household animals is of primary importance in both economic and public health point of view.

Medicinal plants have been extensively used in animal husbandry since ancient times. The crude extracts were directly used as an ailment for many of the symptoms. This was successful for many of the cases but not for all and there was no transparent explanations and scientific documentation on the capability of the plant in curing. The side effects and impact of the treatment on the animal were of less concern and might have led to the loss of production and other complicated diseases.
Terminalia chebula is one of the medicinally exploited plants with many inherent properties. It belongs to the family Combretaceae and is commonly called as Black myrobalan, Ink tree (or) *Chebulic myrobalan*. It is extensively used in Unani, Ayurveda and homeopathic medicine for treatment of neurologic problems, heart disease, inflammation, brain dysfunction, ophthalmic, skin itching and edema in humans. This plant also has adrenergic and stress-reducing functions. *Terminalia chebula* is also used in the field of animal health maintenance.
In 2012 [1] reports shown that antibacterial effect of *Terminalia chebula* against mastitis field isolates. Mastitis is a multi-factorial disease of the dairy cows with a worldwide occurrence and serious health and economic concern in the dairy industry. Mastitis cause Color change, milk yield and alters milk composition. Amongst cattle diseases, bovine mastitis is a serious problem which affects the basic income of the farmers, depleting their daily sources. This condition has to be addressed in rural areas like Wayanad District in Kerala where agriculture and animal cultivation are the major occupation. Since closely associated with natural treatment strategies are also evident in these kinds of hilly places. Traditional practitioners suggest using *Terminalia chebula* for the treatment of mastitis in dairy animals in the district. Recent studies has reported the antimicrobial activity of *Terminalia chebula* against mastitis isolates [2], but some of the oral literature among dairy farmers in Wayanad District, Kerala and studies stated that excessive use of *Terminalia chebula* possess toxic effect and also reduce the yield of milk. Hence our present study is to investigate the cytotoxic effect of *Terminalia chebula* by *in vitro* methods.

Materials and methods

Collection of Medicinal Plants and extract preparation

The study was aimed to screening the *in vitro* cytotoxic effect of *Terminalia Chebula* on L- 929 fibroblast cell lines. The collection and extract preparation was carried out in the Department of Microbiology, Pazhassiraja College, Wayanad, Kerala from the period of January to March 2017. Based on the oral literature collected from farmers *Terminalia Chebula* were selected and authentically identified by Vanamoolika Herbal Research Foundation Wayanad for further studies. The extract was prepared using distilled water as the solvent [3].

***In vitro* cytotoxic determination by MTT assay-** The cytotoxic effect of *Terminalia chebula* by *in vitro* methods on L-929 fibroblast cell line was studied in Biogenix research Centre, Trivandrum, Kerala in order to determine the cell viability after plant extract introduction that measures membrane integrity and effect of the plant extract on cell growth.
The cell line (L929) was obtained from National Centre for Cell Science (NCCS), Pune, India and grown in Dulbeccos modified Eagles Medium (EMEM) containing 10% fetal bovine serum (FBS). The cell line was cultured in 25 cm^2 tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate and antibiotic solution containing: Penicillin (100 U/ml), Streptomycin (100 µg/ml), and Amphoteracin B (2.5 µg/ml). Cultured cell lines were maintained at 37^0C, 5% CO_2, 95% air and 100% relative humidity. Maintenance cultures were passaged weekly, and the culture medium was changed twice a week. [4, 5]

Cell treatment procedure- Two days old confluent monolayer of cells were trypsinized and the cells were suspended in 10% growth medium, 100µl cell suspension ($5x10^4$ cells/well) was seeded in 96 well tissue culture plate and incubated at 37ºC in a humidified 5% CO_2 incubator. One mg of *Terminalia chebul*a Lyophilized powder was added to 1ml of DMEM and dissolved completely by cyclomixer. After that, the extract solution was filtered through 0.22 µm Millipore syringe filter to ensure the sterility. After 24 hours the growth medium was removed, freshly prepared plant extracts in 5% DMEM were five times serially diluted by two fold dilution (100 µg, 50 µg, 25 µg, 12.5 µg, 6.25 µg in 100 µl of 5% MEM) and each concentration of 100 µl were added in triplicates to the respective wells and incubated at 37ºC in a humidified 5% CO_2 incubator. The viability of cells was evaluated by direct observation of cells by Inverted phase contrast microscope and followed by MTT assay method.

Cytotoxicity Assay by direct microscopic observation- The entire plate was observed at an interval of each 24 hours; up to 72 hours in an inverted phase contrast tissue culture microscope (Olympus CKX41 with Optika Pro5 CCD camera) and microscopic observation were recorded as images. Any detectable changes in the morphology of the cells, such as rounding or shrinking of cells, granulation and vacuolization in the cytoplasm of the cells were considered as indicators of cytotoxicity.

Cytotoxicity Assay by MTT Assay Method- Fifteen mg of MTT (Sigma, M-5655) was reconstituted in 3 ml PBS until completely dissolved and sterilized by filter sterilization. After 24 hours of the incubation period, the sample content in wells was removed and 30µl of reconstituted MTT solution was added to all test and cell control wells, the plate was gently shaken well, then incubated at 37ºC in a humidified 5% CO_2 incubator for 4 hours. After the incubation period, the supernatant was removed and 100µl of MTT Solubilization solution (DMSO was added and the wells were mixed gently by pipetting up and down in order to solubilise the formazan crystals. The absorbance values were measured by using micro plate reader at a wavelength of 570 nm.[10] The percentage of growth inhibition was calculated using the formula:

% Cell viability = Abs (sample)/Abs (control) x100

Result

The cytotoxic effect of Terminalia *Chebula* extract on L929 fibroblasts cell line was studied in Biogenix research Centre Trivandrum. Higher concentration of leaf extract, the cell viability remained almost same as that in control (Table 1). This suggests that the extract had no toxic effect on viability of cells.

The % Cell viability (MTT Assay) was determined by using the following formula-

% Cell viability = Abs (sample)/Abs (control) x100

Table 1: Cytotoxic activity of *Terminalia chebula* extracts by *in vitro* methods
LD 50 value – 210.661µl/ml

Sample volume (µl)	Average OD at 540nm	Percentage viability
Control	1.6963	
Sample – C		
6.25	1.6093333	94.87316
12.5	1.5130333	89.19609
25	1.3997667	82.51882
50	1.3037333	76.85747
100	1.2627333	74.44045

Discussion

Terminalia chebula is an herbal tree with invaluable medical applications. *Teminalia chebula* is well-known drugs which prevent aging and imparts longevity, immunity and body resistance against disease and also used extensively in several Ayurveda formulations prescribed for infectious disease. The indigenous herbal medicinal practitioners especially in rural areas recommended for the use of *Terminalia chebula* to dairying farmers for treating mastitis infections in cows. *Terminalia chebula* holds good antimicrobial activity against mastitis isolates [2] but some of the literature stated that excessive use of *Terminalia chebula* possess toxic effect and also reduce the yield of milk.

In-vitro cytotoxic effect of *Terminalia chebula* using methanolic extracts and reported that extracts of *Terminalia Chebula* have got the intense cytotoxic effect and have a potential use in medicine [7]. In another study in 2013 based on the Antioxidant, cytotoxic and analgesic activities of the methanolic fruit extract of *Terminalia Chebula* and results reported that the fruit extract showed

moderate cytotoxic activity with an LC50 OF 97.36 µg/ml [8]. L929 fibroblast cells were metabolically active when treated with aqueous extract of *Terminalia Chebula* at a higher concentration of 100 µl/ml. The response of both cell lines to various extracts and their percentage change in metabolic activity was different depending on the extract type which can be due to the selective cellular response and affinity to compounds in these bioactive extracts [9]. In our present work, the aqueous extract of *Terminalia Chebula* exhibited no cytotoxic effect on L929 fibroblast cells and cells were metabolically active at higher concentrations with LD50 value of 210.661 µl/ml.

Conclusions

The result from this preliminary study indicates that these plant extracts could be used for the therapeutic purpose in case of mastitis which could become serious when secondary infections in mammary glands are caused. The cutaneous and subcutaneous might be treated with the extract of *Terminalia chebula*. Further investigations are needed for identification and purification of the specific antimicrobial and antioxidant compounds from these plants against mastitis pathogens.

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Contribution of Authors

Significant assistances to the idea or design of the work and written part of the manuscript was completed by the corresponding author, reaming sample collection and methodology for the work completed by remaining authors. Perilous modification and final editing of this article were contributed by each author.

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