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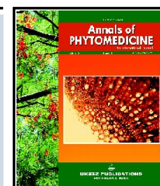

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Evaluation of cytotoxicity of *Azadirachta indica* A. Juss., *Curcuma longa* L. and *Ocimum sanctum* L. in BHK-21 cell line

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Abstract

Curcuma longa L. (Turmeric), *Azadirachta indica* A. Juss. (Neem) and different varieties of *Ocimum* spp. (Tulsi) have demonstrated their antiviral activity against different viruses. The present study was undertaken to evaluate the cytotoxicity of the hydroethanolic plant extract of *A. indica*, *C. longa* and *O. sanctum* in Baby Hamster Kidney cell line (BHK-21 cell line). Hydroethanolic extract (70:30) was prepared from dried and grinded tuber of *C. longa*, leaves of *A. indica* and *O. sanctum*. Cytotoxicity effect of plant extracts and nanoparticles were conducted by dye exclusion method using trypan blue. The CC_{50} was analyzed from liner regression equation of the per cent viable cell within the concentration range of 1.95-1000 µg/ml. The average 50% cell cytotoxicity (CC_{50}) of the hydroethanolic extract of *A. indica* (208.10 ± 1.9 µg/ml) and *O. sanctum* (383.08 ± 1.14 µg/ml) showed lower cytotoxicity in BHK-21 cells in comparison to *C. longa* extract (85.14 ± 1.00 µg/ml) which reveals *A. indica* and *O. sanctum* are less cytotoxic to BHK-21 cell line compare to *C. longa*.

1. Introduction

The probable toxicity of plant extracts and phytochemicals isolated from plants during initial screening, cytotoxicity studies are helpful (McGaw *et al.*, 2014). Moreover, cell cytotoxicity studies are bioassay or screening procedure that is carried out to evaluate *in vitro* cell growth, cell proliferation and morphological changes produced by therapeutic substances. There were several reports that many traditional medicinal plants possess strong antiviral activity with less cytotoxic effect against many organisms that affects human beings (Lin *et al.*, 2014) as well as animals (Yasmin *et al.*, 2020). *C. longa* (Turmeric), *A. indica* (Neem) and different varieties of *Ocimum* spp. (Tulsi) are being used traditionally in India as household remedies to prevent and treat a variety of ailments. Numerous studies have demonstrated their antiviral activity against different viruses like Dengue (Ichsyani, 2017; Parida *et al.*, 2002), FMD (Deshpande and Chaphalkar, 2013), new castle disease (Jayati *et al.*, 2013), *etc.* Various reports are available on toxic effect of extract of neem leaves in specific concentration in experimental laboratory animals and baby hamster kidney fibroblast (BHK-21) (Qurni *et al.*, 2017). Concentration of 10 mg/ml of ethanolic extract of Curcuma inhibited the cancerous cell growth in BHK-21 cells where the growth was inhibited within 24 h and the cellular pathological changes were cell clumping, detachment of cell monolayer, distortion of cellular structure and cell death (Joshi, 2013). *O. basilicum* has showed the cytotoxicity effects on human breast cancer cell line (MCF-7), producing cell death and stimulating mTOR/Akt/p70S6K pathway (Torres *et al.*, 2018). However, there are scanty reports of cell cytotoxicity of hydroethanolic extract of these plants in BHK-21 cell line. Thus, the present study was undertaken to evaluate the cytotoxicity effect of the hydroethanolic plant extract of *A. indica*, *C. longa* and *O. sanctum* in BHK-21 cell line.

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2. Materials and Methods

The fresh leaves of the plant (*Azadirachta indica* A. Juss. and *Ocimum sanctum* L.) were collected from College of Veterinary Science, Khanapara, Guwahati, Assam campus and fresh rhizome of *Curcuma longa* L. were procured from local market (Beltola, Guwahati, Assam). The selected plants were sent for authentication to Botanical Survey of India, Laitumkhrah, Shillong, Meghalaya. Leaves were washed using tap water followed by distilled water to remove the dusts and then shed dried. The dried leaves were then grinded separately and the powder of these plants were stored in glass sealed containers at room temperature (15-25ºC) in cold dark place. Similarly, the rhizomes of *C. longa* were also washed with tape water and then shed dried. The dried rhizomes were milled to obtain powder and store at room temperature (25-30ºC) in a cold dark place till extraction process was carried out.

Hydroethanolic plant extract (Figure 1) of dried plant leaves of *A. indica, O. sanctum* and powdered rhizomes of *C. longa* were made in 70 % ethanol and 30 % distilled water. Fifty grams (50 g)

of plant leaves powder of *A. indica* and *O. sanctum* and powdered rhizomes of *C. longa* were mixed with 500 ml of 70:30 hydroethanolic solvent in separate conical flasks and kept for 24 h at room temperature (25-30ºC) with occasional shaking. The mixture was filtered through muslin cloth three times and then filtrate by using Whattman No. 1 filter paper, after which a clear filtrate was obtained. The filtrates were evaporated by rotary evaporator to obtain the residue and kept in refrigerator (2-8ºC) till further use.

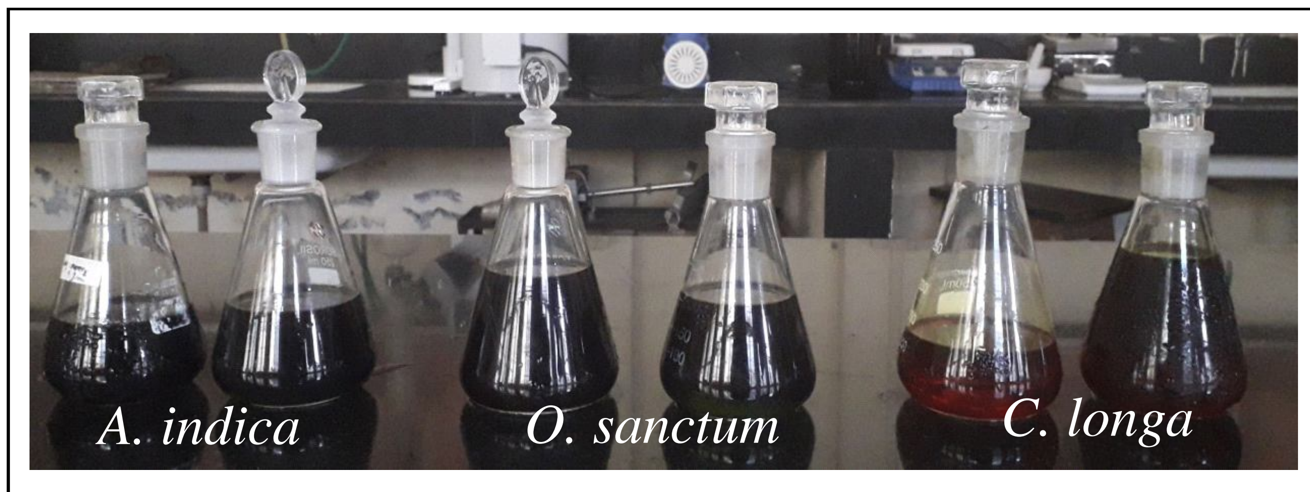

Figure 1: Hydroethanolic extract of *A. indica, O. sanctum* and *C. longa*.

In the present study, Baby Hamster Kidney (BHK-21) cell line (passage no. 10) was obtained in 2019 from the repository of the Regional Research Centre, Indian Council of Agricultural Research (ICAR)-All India Coordinated Research Project (AICRP) on Foot and Mouth Diseases (FMD), Department of Microbiology, College of Veterinary Science, AAU, Khanapara, Guwahati-781022, Assam, India.

Minimum essential media with Dulbecco's Modified Eagle Medium (DMEM), supplemented with 5-10% fetal bovine serum (FBS, Sigma Aldrich) was used to propagate the cell line. Antibiotic solution (containing combination of penicillin, streptomycin, amphotericin B) was added to the growth medium @ 100 units per ml to prevent bacterial contamination. A maintenance medium containing DMEM with 5% FBS and 100 units per ml antibiotic was used to maintain the BHK-21 cell monolayer.

BHK-21 cells with 90-100% confluent monolayer were used for subculturing in cell culture flask of capacity of 25 ml tissue culture polystyrene nunc plate (Becton Dickinson, USA). After discarding the growth media, BHK-21 monolayer cells were washed and added with DMEM to 25 ml cell culture flasks and 500 µl of 0.25% trypsin with 0.2% EDTA solution (Himedia®, India) was added. The cell culture flasks were kept for 2-3 min at room temperature and trypsin was discarded and the monolayer cells were detached by gently tapping the flask. The cells were split in 1:3 ratio from 25 ml cc flasks.

In the study, cytotoxicity effect of plant extracts was evaluated by dye exclusion method using trypan blue as per Tolo *et al.* (2006). BHK-21 were taken in 96 well tissue culture plates and incubated for 48 h at 37°C in 5% CO_2 atmosphere. The medium was removed from the wells and 100 µl of dilutions of the test compounds (1000, 500, 250, 125, 62.5, 31.25, 15.63, 7.81, 3.91, 1.95 µg/ml concentration) prepared in cell culture medium (DMEM), were added to each well. Control cells were added with only 100 µl of the medium. The plates were then put in incubator for 3 days under similar condition as stated above. After 72 h, the cells were trypsinized and were stained with 0.4% trypan blue with 1:2 dilution (50 ml stain and 50 ml maintenance media). Cells were counted using hemocytometer in the big 4 primary squares. Live cells looked transparent (without stain) and dead ones looked blue (stained) (Figure 2). Per cent viability of cells were calculated by:

Total cells/ml = Total cells in a square × dilution factor × 10^3

Viable cells/ml = Viable cells in a square × dilution factor × 10^3

% of viable cells = 100 × (viable cells / total cells)

The 50% cytotoxic concentration (CC_{50}) was estimated from the linear regression graph by plotting % cell viability against concentration of the test compounds (1.95-1000 µg/ml).

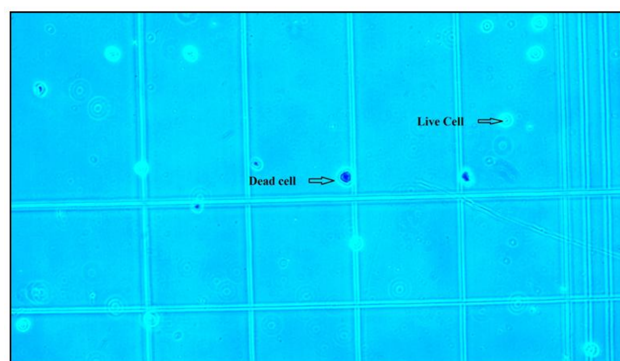

Figure 2: Live (unstained) and dead (stained) BHK-21 cells in typan blue staining.

The cytotoxic effect was observed in the BHK -21 cell line in the form of cell rounding, granulations, loss of intracellular matrix and detachment of the cells from the surface. The CC_{50} was analyzed from linear regression equation of the per cent viable cell within the concentration range.

All the experiments were performed in triplicate. The graphic data was presented as Mean ± S.E. The p value < 0.05 was considered significant using ANOVA. CC_{50} was calculated by linear regression analysis from the intercept in the slope of linear best fit line and X is the corresponding concentration at which Y occurred.

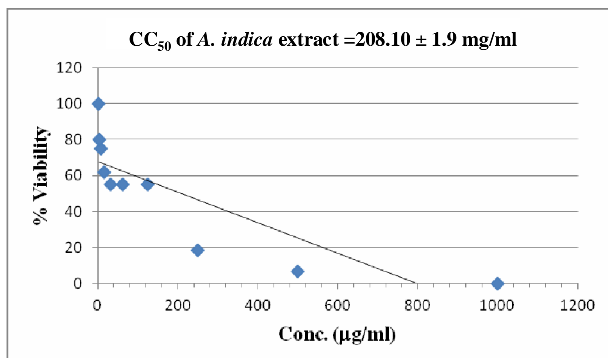

Figure 3: Linear regression graph of CC_{50} of *A. indica* extract in BHK-21 cell line.

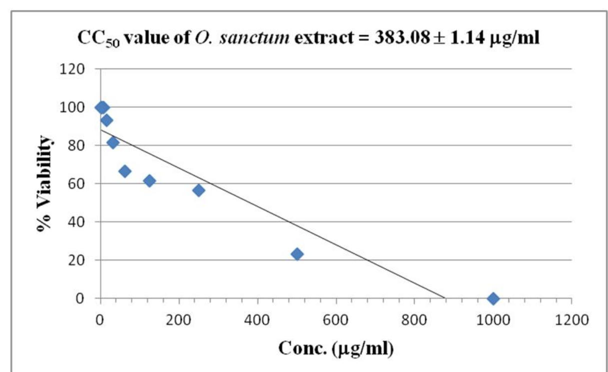

Figure 4: Linear regression graph of CC_{50} of *O. sanctum* extract in BHK-21 cell line.

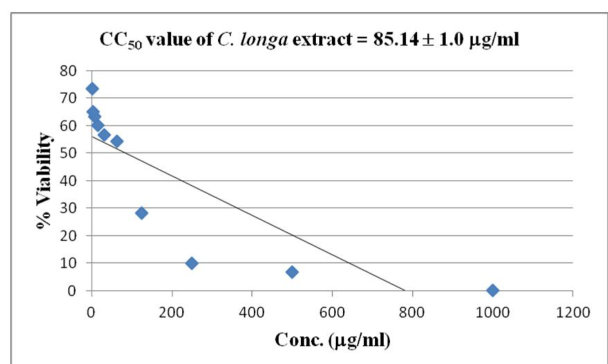

Figure 5: Linear regression graph of CC_{50} of *C. longa* extract in BHK-21 cell line.

3. Results

In the present study, the CC_{50} was analyzed from linear regression equation of the per cent viable cell within the concentration range of 1.95-1000 µg/ml for various extracts of studied plant materials. The average 50 % cell cytotoxicity (CC_{50}) of hydroethanolic extract of *A. indica*, *O. sanctum* and *C. longa* in BHK-21 cell line was found to be 208.10 ± 1.9 µg/ml (Figure 3), 383.08 ± 1.14 µg/ml (Figure 4) and 85.14 ± 1.00 µg/ml (Figure 5), respectively. The CC_{50} values of hydroethanolic extracts of *A. indica*, *O. sanctum* and *C. longa* observed in the present study differed significantly ($p<0.05$) from each other. The hydroethanolic extract of *A. indica* (208.10 ± 1.9 µg/ml) and *O. sanctum* (383.08 ± 1.14 µg/ml) showed lower cytotoxicity in BHK-21 cells in comparison to *C. longa* extract (85.14 ± 1.0 µg/ml).

4. Discussion

The average 50 % cell cytotoxicity (CC_{50}) of hydroethanolic extract of *A. indica* in BHK-21 cell line was found to be 208.10 ± 1.9 µg/ml. Reports are available for antiviral activity of different plant extracts against FMD virus in BHK-21 cell line and found the CC_{50} value of 200 µg/ml and 50-400 µg/ml in respect of ethanolic and aqueous extracts of *A. indica*, respectively (Imran *et al*., 2016; Younus *et al*., 2017). The CC_{50} value of methanolic extract of *A. indica* in vero cell line was reported to be 8000 µg/ml in another study (Joe *et al*., 2019). Active components of neem have been evaluated for their apoptotic effects in many human cancers, especially leukaemia (Kikuchi *et al*., 2011), cervical (Mahapatra *et al*., 2011), prostate (Priyadarsini *et al.,* 2010) and breast (Elumalai *et al*., 2012). Neem produces a cascade of molecular events that are related with apoptotic cell death and increase in the expression of bim, bax, apaf-1, caspase 8, caspase 3, PARP cleavage and also reported suppression of Bcl-2 expression (Balasenthil *et al*., 1999; Subapriya *et al*., 2005). Active components of neem, azadirachtin and nimbolide inhibit phase 1 (carcinogen activation) enzymes and induce activation of phase 2 (carcinogen detoxification) enzymes that helps to control the early phase carcinogenesis (Priyadarsini *et al*., 2009).

The CC_{50} value of hydroethanolic extract of *O. sanctum* in BHK-21 cells in the present study was found to be 383.08 ± 1.14 µg/ml. Reported study revealed the CC_{50} value of 110 µg/ml of dichloro-methan and methanolic extracts of *O. sanctum* in vero cell line (Yucharoen *et al*., 2011). In another study, Jadhav *et al*. (2012) found the CC_{50} value of methanolic extract of *O. sanctum* in vero cell line to be 278.5 µg/ml. Higher CC_{50} values of 1886.7 ± 1.5 µg/ml and 3849.3 ± 0.5 µg/ml in respect of aqueous and ethanolic extracts of *O. sanctum*, respectively were also reported (Bhanu-prakash *et al*., 2008). Essential oils of *Ocimum* spp. contains monoterpene derivatives, *e.g.*, camphor, thymol, limonene, geraniol, citral, and linalool and phenolic compounds, *e.g.,* cinnamic acid, sinapic acid, caffeic acid, rosmarinic acid and ferulic acid (Lawrence, 1993; Loughrin and Kasperbauer, 2001). These compounds act as potent antioxidants, electrophile scavengers, metal chelators, induce detoxification of enzymes and inhibit the formation of DNA addicts with carcinogens (Beliveau and Gingras, 2007). The cytotoxic activity of *Ocimum* spp. may be attributed to antioxidant potential of these polyphenolic compounds present in the extracts.

The average 50% cell cytotoxicity (CC_{50}) of hydroethanolic extract of *C. longa* in BHK-21 cell line in this study was found to be 85.14 ± 1.00 µg/ml. The MNCT of 5 µg/ml and 0.62 µg/ml in respect of ethanolic and ethyl acetate extracts, respectively of *C. longa* in BHK-21 cells was studied (Ashraf *et al*., 2019). It has been reported earlier the antiviral effects of *C. longa* against dengue virus *in vitro* in Huh 7it-1 cell line and found the CC_{50} of ethanolic extract to be 85.4 µg/ml (Ichsyani *et al*., 2017). In an alternative study (Wahyuni *et al.,* 2018) reported the CC_{50} of ethanolic extract of *O. sanctum* in

Huh7it-1 cell line to be >100 µg/ml. Curcumin is the major active ingredient of *C. longa*, that has been reported to be an effective phytochemical for cancer prophylaxis and therapy. Curcumin has been shown to have antiproliferative effect in normal cells and can reduce cell viability in cell culture studies (Soleimani *et al.*, 2018). Cell death, autophagy, and arrest of cell cycle has been demonstrated by the use of curcumin (Zhu and Bu, 2017). *In vitro* and *in vivo* studies have revealed that curcumin targets many anticancer signalling pathways (*e.g.,* p53, AKT, Wnt/β-catenin, Ras, phosphoinositide 3-kinase) (Kasi *et al*., 2016) and turmeric was also reported to modify regulation of micro-RNAs network expression (Mirzaei *et al.*, 2018) and inhibits histone deacetylases activity (Soflaei *et al*., 2018).

In the present study, CC_{50} values of hydroethanolic extracts of *A. indica* (208.10 ± 1.9 µg/ml) and *O. sanctum* (383.08 ± 1.14 µg/ml) showed lower cytotoxicity in BHK-21cells in comparison to *C. longa* extract (85.14 ± 1.00 µg/ml). Variation in the value of CC_{50} found in different studies might be attributed to the difference in the type of extract, type of solvent used in the extraction procedure, cell line, medium and number of passages. Moreover, the phytochemical constituent of the plants plays vital role in cell viability and variations may occur due to the variation in the quantity and types of cytotoxic substances (*e.g*., alkaloids, flavonoids, steroids, gums and carbohydrates, reducing sugars, saponins, tannins, *etc*.) present in the extracts.

5. Conclusion

In the present study, it was observed that the hydroethanolic extract of *A. indica*, *O. sanctum* showed better cell survivality of BHK-21 cell line in comparison to *C. longa*. The result of the present study may be used for *in vitro* cell proliferation and cytotoxic studies in cell culture of these plant extracts. However, this cell cytotoxicity of the crude plant extracts in BHK-21 cells is suggestive of the presence of potent cytotoxic compounds which warrants further investigation.

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Conflict of interest

The authors declare that there are no conflicts of interest relevant to this article.

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