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Research Article

EFFECTS OF *CENTELLAASIATICA* L., *CURCUMA LONGA* L., AND *STROBILANTHESCRISPUS* L. EXTRACTS ON 3 KIDNEY CELL LINES: *IN VITRO* CYTOTOXICITY ANALYSIS

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ABSTRACT

Objective: This study was carried out to evaluate the *in vitro* cytotoxicity to three cell kidney lines by using the 3-(4, 5-d imethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) reduction assay of three popular medicinal plants used in Malaysia.

Methods: Methanol and aqueous extracts of *Centellaasiatica* L., *Strobilanthescrispus* L. and *Curcuma longa* L. were tested at the non-toxic limit concentration at 50 ($NTLC_{50}$) ranging from 50 µg/ml and 200 µg/ml depending on the cell lines used, i.e. African Green Monkey Kidney (Vero), Baby hamster Kidney (BHK) and Rabbit Kidney (RK) cells.

Results: *Centellaasiatica* L. was the least toxic to the all cell lines tested followed by *Strobilanthes crispus* L. and *Curcuma longa* L. Methanol plant extracts inhibited cell growth but not to the aqueous plant extracts. Meanwhile, BHK cells were found to be the most resistant to the plant extracts.

Conclusion: This study proves the safety of these plant extracts for future scientific studies in its biomedical properties.

Keywords: *Centella asiatica* L., *Curcuma longa* L. and *Strobilanthes crispus* L., cytotoxicity

INTRODUCTION

The medicinal values of herbs were normally based on tradition and accidental discovery. These practices are potentially toxic or harmful [1,2]. Indeed, it is difficult to measure these systemic effects *in vivo*. Cytotoxicity testing is important for the sole purpose of determining the potential toxicity of the compounds being studied. Cytotoxicity is cell damage but the definition somehow is varying depending on the nature of the study whether cells are killed or their metabolism altered. It is a complex event *in vivo* and could be direct cellular damage, physiological effects, systemic effects, inflammatory effects and other systemic effects [3]. Cytotoxic agents unselectively kill and damage both normal and cancerous cells by interfering with either, the cellular process or mechanical process. The unspecific action of cytotoxic agents constitutes a major drawback in any therapy especially cancer chemotherapy [4]. is different with anticancer agents which are designated to kill the cancerous cells where cytotoxicity is crucial to the action [3].Currently, the non-radioactive, calorimetric assay system using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT) has been widely used for evaluating cell viability *in vitro*. The assay measures the conversion of MTT into purple-colored MTT formazan by living cells and decrease in cellular MTT reduction could be an index of cell damage [5]. There were numbers of studies dealing with cytotoxicity screening of plant extracts [6-8] but mostly tested in cancerous cells rather than in normal cell cultures. Cytotoxicity studies with normal culture systems (tissue culture) of local plant extracts or folk medicinal plant extracts has not been studied extensively and this is vital for the safety evaluation or any herbal preparations [9].Thus, the objective of this study was to evaluate the potential cytotoxicity of methanol and aqueous extracts of *Centella asiatica* L.,*Curcuma longa L.* and *Strobilanthes crispus* L. in normal kidney animal cell lines (Vero, BHK and RK cells). *Centellaasiatica* L. (Umbelliferae) is a small herbaceous plant that originally found in swampy areas of India and Tropical Island of the Indian Ocean[10,11]. It is commonly consumed fresh as vegetable (salad) among Malay communities [12], as cooling drink by Chinese and brain tonic by the Indian people [13]. In Malaysia, the plant is commonly known as 'pegaga'.*Strobilanthes crispus*L. Bremek (Acanthaceae) is native to countries from Madagascar to Indonesia [10]. In Malaysia, the plant is commonly known as 'daun picah beling' whereas in Java it is called by 'enyohkelo', 'kecibeling' or 'kejibeling'[9, 14].*Curcuma longa* L. (Zingiberaceae) has long been considered an essential flavoring spice of Indian and other ethnic cuisines. Plants of the *Curcuma* genus, especially *Curcuma longa* are tropical plants, which is originated in Asia (probably India) and it is cultivated extensively throughout the warmer parts of the world primarily in Bengal, China, Taiwan, Sri Lanka, Java, Peru and Australia [10, 12]. All these three plant species have been reported to possess lots of medicinal properties. Some of their medicinal propertiesare presented in Table 1.

MATERIALS AND METHODS

Plant collection

The rhizomes of *Curcumalonga* L. was obtained from Sungai Buloh, Selangor, West Malaysia while the two other herbs; the leaves of *Centellaasiatica* L. and Str*obilanthescrispus* L. were obtained from a farm in BatuPahat, Johore, West Malaysia. The botanical identification of collected plants was done and voucher specimens are conserved at the Phytomedicinal Herbarium, Institute of Bioscience, Universiti Putra Malaysia.

Preparation of plant extracts

The samples were washed thoroughly before air-dried for 24 hrs. Then, they were cut into slices and ground into powder. The aqueous extracts of *Curcumalonga* L. rhizomes, *Centellaasiatica* L. leaves and *Strobilanthescrispus* L. leaves were prepared as described by Zakaria et al. [17] and Adam et al.[18]. The samples also underwent methanol extraction as described as Salleh et al. [19] and Zakaria et al., [20].

Cell cultures and incubations

The cell lines used in the study were Vero cells Vero cells (ATCC No. CCL-81), BHK cells (ATCC No. CRL-10314)and RK cells (ATCC No.CCL-106). These established cell lines were grown in 25 cm^2 sterile disposable polystyrene cell culture flask (TPP). The Vero, BHK and RK cells were cultivated using RPMI medium (preparation 1640 (GIBCO BRL®)) (Powder with L-Glutamine without Sodium Bicarbonate) and EMEM (Eagle's Minimal Essential Medium) preparation with EarlesSalts with L-Glutamine (Flowlab), respectively, supplemented with 4% (v/v) Fetal Calf Serum (FCS) and 1% (v/v) antibiotic-antimycotic for

growth medium and 1% (v/v) FCS and 1% (v/v) antibiotic-antimycotic solution for maintenance medium. Briefly, the Vero, BHK and RK cells were seeded at $8x10^3$cells per well in 96 well flat-bottomed microtitre plates (TPP) each triplicate. The plates were then incubated in humidified atmosphere (37˚C, 5% CO_2) for 2-3 days for the cells to confluent.

Table 1: Some of the medicinal uses of Centellaasiatica L., Strobilanthescrispus L. and Curcuma longa L.

Plant	Usage
Centellaasiatica L.	Scabies, skin ulcers, diarrhea, red eyes, jaundice, tonsillitis, measles, respiratory problems,peptic ulcer, cataract, leprosy,cholera, fever, dysentery, headache and syphilis.
*Strobilanthescrispus*L.	Diuretic, antidiabetic, antilytic, antihepatic, treat food poisoning and snake bite, lowering blood cholesterol level, anticancer, anti eczema, anti haemorrhoids, anti constipation, laxative and antibacterial.
*Curcuma longa*L.	Bruise, catarrh, dysentery, gonorrhea, smallpox, cholagogueue, colic, depurative, dermatosis, conjunctivitis, antioxidant, scabies, jaundice,anti inflammatory, antiviral and anticancer.

Adaptedfrom [14, 15, 16]

A serial of twofold dilution of the plant extract and a serial of twofold dilution of standard (Cycloheximide), concentrations ranging from 3.13 to 200 µg/ml were prepared in maintenance medium (RPMI medium for Vero cells and EMEM for BHK and RK cells). All preparations were filter sterilized by membrane filter 0.45 µm (Schleicher &Schuell, Germany).

The final concentration of methanol in the cytotoxicity assay did not exceed 0.5% v/v, at which no cytotoxic effect was observed [21].The plates were returned to incubator in humidified atmosphere (37˚C, 5% CO_2) for 3 days.

Estimation of cell viability

The tetrazolium salt, MTT 0.5 mg/ml (Sigma), was diluted in PBS pH 7.2 at room temperature, avoiding from light and filter sterilized. It was added to each of wells (96 wells per plate) at 20 µl (10% of cell volume) and the plate was wrapped in aluminum foil.

The plates then were incubated for 3 hrs in humidified atmosphere (37˚C, 5% CO_2). The old medium and MTT were discarded from the wells after 3 hrs of incubation and 200 µl of Dimethylsulphoxide (DMSO) was added to all of the wells to dissolve the MTT-formazan crystals[22].

The absorbance was recorded immediately at 550 nm and reference wavelength at 650 nm by using ELISA machine [21].

Statistical analysis

Results were expressed as mean ± standard deviation of triplicate experiments. Statistical significance was determined by analysis of variance using SPSS 19.

RESULTS AND DISCUSSION

The non-cytotoxicity was expressed as the 50% non-toxic limit concentration ($NTLC_{50}$), which is the concentration of plant extract to sustain the growth of cells up to 50%. The $NTLC_{50}$ of the three plants were ranging from 50µg/ml and 200 µg/ml depending on the cell type used. The mean percent (%) cell viability of three different plants, which were *Centella asiatica* L., *Curcuma longa* L. and *Strobilanthes crispus* L. was significantly different ($P \leq 0.05$). *Centella asiatica* L. has showed better mean percent (%) cell viability (84.86 ± 32.99 %) than the mean percent cell viability of *Strobilanthes crispus* L. (83.98 ± 21.08 %) and*Curcuma longa* L. (73.17 ± 19.19 %) (Table 2).Besides, two different ways of extraction, which were methanol extraction and aqueous extraction, did have significant effect on the mean percent (%) cell viability (76.66 ± 29.26% vs. 84.68 ± 20.78%, respectively) (Table 3). The sensitivity of various types of cell also tested and has found that they were significantly different with the mean percent (%) cell viability of BHK cells was 87.66 ± 26.93% better than the mean percent (%)Vero cell viability (82.85 ± 26.83%) and RK cell viability (71.50 ± 20.06%) (Table 4).

Table 2: Mean of percent (%) cell viability for plants

Plants	Percent (%) cell viability
Centellaasiatica L.	84.86 ± 32.99^a
Strobilanthescrispus L.	83.98 ± 21.08^a
Curcuma longa L.	73.17 ± 19.19^b

Values are mean ± S.D. (%) of triplicate experiments regardless of plant extract concentration, methods of plant extraction and cell types used. Mean with the same superscript (a, b and c) are not significant at P =0.05.

Table 3: Mean of percent (%) cell viability for different methods of plant extraction

Methods of extraction	Percent (%) cell viability
Methanol extract	76.66 ± 29.26^a
Aqueous extract	84.68 ± 20.78^b

Values are mean ± S.D. (%) of triplicate experiments regardless of plant extract concentration, plant species and cell types used. Mean with the same superscript (a, b and c) are not significant at P =0.05

Table 4: Mean of percent (%) cell viability for cells

Cells	Percent (%) cell viability
BHK	87.66 ± 26.93^a
Vero	82.85 ± 26.83^b
RK	71.50 ± 20.06^c

Values are mean ± S.D. (%) of triplicate experiments regardless of plant extract concentration, methods of extraction and plant species. Mean with the same superscript (a, b and c) are not significant at P =0.05.

Non-cytotoxicity level of plant extracts and sensitivity of cells

Centellaasiatica L. leaf Extracts

The methanol leaf extraction of *Centella asiatica* L. was found to have $NTLC_{50}$ on percent (%)Vero, BHK and RK cells viability ranging from 100-200 µg/ml, 200µg/ml and 100µg/ml, respectively. When compared with aqueous leaf extraction, it showed that the $NTLC_{50}$ on percent (%) cell viability for three cells was much higher than methanol leaf extraction (at concentration 200 µg/ml and above).

Curcuma longa L. rhizome Extracts

All the methanol extract of *Curcuma longa* L. rhizome showed the same $NTLC_{50}$ to Vero, BHK and RK cells, which was 100µg/ml. The BHK cells showed greater mean percent (%) cell viability (107.49±28.34%) when tested at the lowest concentration of ME, compared to Vero (100.24±5.95%) and RK (85.80±2.02%). The cytotoxicity effect was dose dependent, which higher concentration of ME decreased cell viability.

Similar trend was showed in the AE of *Curcuma longa* L. rhizome where the cytotoxicity effect was dose dependent, with BHK has the highest mean percent (%) viability but the $NTLC_{50}$ to Vero, BHK and RK cells was dissimilar to ME, which was 200 µg/ml, 50 µg/ml, and 200 µg/ml, respectively.

Strobilanthes crispus L.

All methanol *Strobilanthes crispus*L.leaf extracts showed $NTLC_{50}$ to RK, BHK and Vero cells above 200 µg/ml with significant different of mean percent (%)cell viability (130.34 ± 9.22% vs. 111.90 ± 27.76% vs. 65.57 ± 2.69%), respectively. Likewise, the AE of *Strobilanthes crispus* L. also showed $NTLC_{50}$ to cells above 200 µg/ml but with dissimilar type of cells, which BHK cellswere more resistant than RK and Vero cells (102.63 ± 9.55% vs. 53.71 ± 0.44% vs. 52.33 ± 5.04%), respectively (Table 5).

Cycloheximide (Control group)

Cycloheximide ($C_{15}H_{23}NO_4$) treatment was used as positive control of the cells. As a potent protein synthesis inhibitor, at concentration of 5mg/ml, it caused 30% apoptosis to neutrophils, lymphocytes and polymorphonuclear cells in the lamina propria of the small intestine at 4-6 hr of incubation period. However, at 50 µg/ml, Cycloheximide,did not cause significant toxicity to cell lines[23].

From the result, Cycloheximide was not significantly toxic to cell cultures within concentrations of 3.13 to 200 µg/ml. The lowest mean percent cell viability toCycloheximide was 48.99 ± 0.76 at the highest concentration, (200 µg/ml in RK cells). The BHK and Vero cells have showed to be less sensitive to induction of apoptosis and have greater $NTLC_{50}$ compared to RK cells

Table 5: $NTLC_{50}$ of methanol and aqueous extract of *Centellaasiatica* L., *Curcuma longa* L. rhizome and *Strobilanthescrispus* L. in BHK, Vero and RK cells.

Plants	Methanol Extract (µg/ml)	Aqueous Extract (µg/ml)
Centellaasiatica L.		
BHK	> 200	> 200
VERO	100-200	> 200
RK	100	> 200
Curcuma longa L.		
BHK	100	50
VERO	100	200
RK	100	200
Strobilanthescrispus L.		
BHK	> 200	> 200
VERO	> 200	> 200
RK	> 200	> 200

CONCLUSION

Methanol extracts (ME) were, as expected generally more cytotoxic than aqueous extracts (AE), which showed that methanolic plant extracts of *Centella asiatica* L., *Curcuma longa* L. and *Strobilanthes crispus* L. have the $NTLC_{50}$ lower (76 µg/ml) than aqueous plant extracts (85 µg/ml). The results are consistent with Serkedjievaand Ivancheva,[6]; Choi and Hwang, [21];Bezivin et al., [24]; Rajbhandari et al., [25]and Tan et al., [26], by which the methanolic plant extracts inhibited cell growth, even at a lower concentration, which was less than 20 µg/ml. In addition, these findings were inclined with studies by Barrio et al., [8]; Chiang et al., [15];Kott et al., [27]and Wattanapitayakul et al., [28], by which AE of Argentine medicinal plants, *Phyllanthus orbicularis*, *Plantago major* L., and *Curcuma longa* L. , respectively showed no cytotoxic effect beyond 400 µg/ml in normal and tumour cell line. The weak cytotoxic activities exhibited by the plants AE may indicate that the compounds present are non-toxic.

The cytotoxicity studies also showed that the extracts impaired the cell viability with respect to the corresponding untreated cultures ($P< 0.05$) [16, 29]. In contrast, the results were not inclined with the findings by Semple et al., [30]and Kuo et al., [31] , by which ethanolic plant extracts of Australian medicinal plants and *Psychotriaserpens*, respectively showed no effect on cell viability up to 200 µg/ml. These discrepancies may not be due the extraction solvents, as the final concentration of the methanol has been standardized to not exceed 0.5% v/v. It is likelythat the plant compounds itself, toxic to cells and affected cell viability. Cytotoxicity may be expressed by vinca alkaloids (plant derivatives), which are capable of specifically affecting the microtubule function and thus impeding the formation of mitotic spindle [32].

In the present study, the ME of *Centella asiatica* L. showed not to cause cytotoxicity at NTLC50up to 150 µg/ml. In contrast, Babu et al.,[33]reported that the ME of *Centella asiatica*L. caused 50% cell death to Dalton's lymphoma ascites tumour cells (DLA) and Ehrlich ascites tumour cells (EAC) at 75µg/ml and 62µg/ml,respectively. This is due to the presence of highly complex of saponins and glycosides that occurred in genus *Centella*, which mostly were toxic [10, 26]. In addition, cancerous cells were more fragile and easily affected rather than normal cell lines[26, 33]. However, studies by Calderon et al., [34]indicated that several methanol Panamanian plant extracts did not possess cytotoxicity effect in cancerous cells at concentration more than 100 µg/ml. By the way, there was no report ME of *Centella asiatica* L. had been tested in normal cell line. Additionally, cytotoxicity assay using Brine shrimp lethality test [35] showed that the ethanol extract of *Centella asiatica* L. did not exert significant cytotoxic activity, though at 1000 µg/ml.

Likewise, the ME of *Strobilanthes crispus* L. did not induce cell toxicity at the highest concentration (200 µg/ml). This result will serve as foundation for upcoming tests regarding the toxicity of cells. In addition, with ascending concentration of the plants ME, the cell viability was increased rather than the plants AE. These findings suggested that the plants ME supported and promoted cell growth.Similar trend was found in the ME of *Curcuma longa* L. rhizome. Itdid not cause significant toxic effects on cell lines.

The results are consistent with Kuo et al., [31];Jiang et al., [36]and Chun et al., [37] which found that Curcumin, a yellow coloring agent from *Curcuma longa* L. rhizome, induces apoptosis in various types of human tumour cell lines, but the compound was inactive for the normal cells in the primary culture.

In addition, Samahaet al., [4]has reported that Curcumin is not toxic to mammals at very high doses (5-10% by weight of diet). However, ethanol extract of *Curcuma longa* L. has reduces cell number at concentration as low as 15 μg/ml in human cervical carcinoma cell line (HeLa cells)[28]. In addition, with ascending concentration of ME, it decreased the mean percent (%) cell viability.

Besides, among three types of cell tested for plant cytotoxicity, the BHK cells were found to be the most resistant cells compared to Vero cells and RK cells. However, the result was not consistent with the results reported by Abad et al., [16]; Kott et al., [27]; and Kuo et al., [31],by which the Vero cells were harder and could sustain the cell growth at plant extracts concentration up to 300 μg/ml. However, the result are consistent with Sekedjieva and Ivancheva[6];Rajbhandari et al., [25] andSemple at al., [30]by which the BHK cells showed to be resistance to the plant extracts than the other two types of cells.

These differences could be due to the different cell cultures of different animal species were used in the cytotoxicity assay, some variation in the way plant compounds acts in the different cell types [38].

The $NTLC_{50}$ of ME and AE, were crucial for future*in vitro* antiviral or anti-microbial tests as the compounds forthe agents must be non toxic to the host cells as the toxic compounds were thereby defeating the selective purpose of the compounds.

As there has been extensive documentation on poor selective toxicity and fast selection of resistant viral variants with the existing drugs such as zidovudine (AZT), dideoxyinosine (ddI) and acyclovir (ACV) [27] the low cytotoxicity of *Centella asiatica* L., *Strobilanthes crispus* L.

And *Curcuma longa* L. reaffirms the suitability of these plant for exploitation as antiviral or antimicrobial agents with selective toxicity, as judged by the criterion set by the National Cancer Institute, which stated that the extracts with $NTLC_{50}$ less than 20 μg/ml were considered to be cytotoxic against the treated cells [26, 39].

Therefore, the screening of the plant extracts toxicity was significant towards more biomedical research. All the medicinal plants tested showed did not cause significant toxic effect in normal cell lines.Thus, merit future biomedical investigations on their numerous biomedical effects.

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