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Original Article

Cell proliferation assessment of human gingival fibroblasts treated with *Clinacanthus nutans* using alamarBlue assay

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Abstract *Clinacanthus nutans* (*C. nutans*), a well-known ethnopharmacological plant consumed for its medicinal purposes by Southeast Asian communities. *C. nutans* is said to possess antipyretic, inflammatory, antiedemic as well as analgesic properties and used traditionally in treating various skin ailments, *Herpes* infection, cancer and diabetes. The young leaves of this *C. nutans* are consumed in Malaysia for maintaining health. In this study, the proliferative activity of human gingival fibroblast cells (HGF-1, ATCC®CRL-2014™, USA) treated with the ethanol extract obtained from *C. nutans* leaves at three different concentrations (250, 125 and 62.5 µg/ml) was compared with the untreated cells using alamarBlue assay. The proliferative activity of HGF-1 using alamarBlue assay showed that the cells treated with 62.5 µg/ml of ethanolic extract of *C. nutans* leaves exhibited increased proliferation compared to the other groups and hence does not exhibit any cytotoxicity on HGF-1.

Keywords: AlamarBlue assay; cell proliferation; *Clinacanthus nutans*; human gingival fibroblast; Sabah snake grass.

Introduction

Popular among Southeast Asians, the ethnopharmacological herbal plant known as *Clinacanthus nutans* (*C. nutans*) or "*Sabah snake grass"* is consumed and used for its medicinal uses. *C. nutans* is a natural remedy in tropical Asia for a long time and is a standout amongst the species from this family. Many researchers have been attracted and fascinated by its medicinal potency on tumour cells (Putwatana *et al.*, 2009) as well as in the treatment for skin inflammation and lesions caused by virus (Wanikiat *et al.*, 2008). AlamarBlue assay is one of the common tests used to study the proliferation of various cell lines and to check the cytotoxicity of chemicals (Fields and Lancaster, 1993). Assessment of cell proliferation and cytotoxicity is an important step which reflects on the cellular health. AlamarBlue assay is an established technique to indicate the cell viability which is based on the reducing power of the living cells in converting resazurin to a resorufin, a fluorescent molecule. Cytotoxicity according to Freshney (2010), is the quality of being toxic to cells which occurs either by way of killing the cells or having their metabolism altered. Cytotoxicity also can be defined as a cascade of molecular events that play a role in interrupting with the macromolecular synthesis which results in an unequivocal functional and structural damage to the cells (Aldridge, 1993). Hence, cytotoxicity assays are necessary to identify the cytotoxic nature of test substances, plant-derived extracts as well as purified compounds that could be intended for pharmaceutical or cosmetic products (McGaw *et al.*, 2014). Identification of common cytotoxicity assays are made based on the measurements of metabolic activity indicating the cytotoxic nature of extracts of plant and its purified compounds (Bunel *et al.*, 2014; McGaw *et al.*, 2014). There have been many claims by researchers regarding the cytotoxicity and anticancer activity of crude extract of *C.*

nutans (Yong *et al.*, 2013; Arullappan *et al.*, 2014; Che Sulaiman *et al.*, 2015; Huang *et al.*, 2016). However, to the best of our knowledge, there is still dearth of information on the proliferative effect of *C. nutans* on human gingival fibroblast cell line (HGF-1). HGF-1 was chosen for the current study as it forms the main cell population of gingival connective tissue and is also involved in the production of extracellular matrix of tissue, both in disease and health (Bartold *et al.*, 2000; Poggi *et al.*, 2003). Therefore, this study aimed to evaluate the proliferation of HGF-1 treated with different concentrations of ethanolic extracts of *C. nutans* leaves from day 1 until day 7 in comparison with the control (untreated cells).

Materials and methods

The leaves of *C. nutans* were obtained from the TKC Herbal Nursery Sdn. Bhd., located in Seremban, Negeri Sembilan, Malaysia. The leaves were cut into small pieces, oven dried at a temperature of 40-45°C for a period of 1-2 days until constant dry weight, following which the leaves were mechanically ground to obtain fine powder. The powder was soaked in ethanol for 3 days following which it was concentrated using a rotary evaporator at 40°C. Human gingival fibroblast cell line (HGF-1, ATCC®CRL-2014™, USA) of passage 17 was used in this study. HGF-1 was grown until confluence in complete media containing alpha modified Eagle's medium (αMEM) (Gibco, Life Technologies, USA) to which was added 10% foetal bovine serum (FBS) (Gibco, Life Technologies, USA), 100 units/ml of 5% penicillin and streptomycin (100 mg/ml) (Gibco, Life Technologies, USA), in a 5% CO_2 incubator at 37°C in a humidified atmosphere. For passaging, the medium was discarded, and Dulbecco's phosphate buffer saline (DPBS) (Gibco, Life Technologies, USA) was used to wash the cells before trypsinization process was carried out using 1 ml of TryplE™ Express (Gibco, Life Technologies, USA), stable trypsin for 25 cm^2 flask. For 75 cm^2 flask, 3 ml was used. The cells were left in the incubator for 5-10 minutes for dissociation of cells to take place. Then, neutralization process of the cells was done using complete media (3 ml in the case of 25 cm^2 and 9 ml for 75 cm^2 flasks). The cell suspensions were centrifuged at a speed of 1200 rpm for 5 minutes following which the complete media (3 ml for the 25 cm^2 flask and 9 ml in the case of 75 cm^2 flask) was discarded. Then, 1000 µl of complete medium was added to the cells. The cells were dispensed at a subculture ratio of 1:3. Then, HGF-1 cells were subcultured with 1 ml of culture medium in a 6-well plate. The cells were now treated with 3 different concentrations (250, 125 and 62.5 µg/ml) of ethanolic leaf extracts of *C. nutans* diluted in complete αMEM and further incubated in a CO_2 incubator at 37°C with 5% CO_2 for a period of 1, 2, 3, 4, 5, 6, and 7 days.

Then, 10% v/v of metabolic indicator dye, alamarBlue® (Invitrogen, Thermo Fisher Scientific, USA) was added both to the wells containing only medium without cells (negative control) as well as to those with cells. Then, 100 µl of solution was taken from each well and transferred to 96-well plate after which the absorbance values were spectrophotometrically measured using an ELISA reader (Tecan, DKSH, Germany) at day 1, 2, 3, 4, 5, 6, and 7 using a wavelength of 570 nm and 600 nm. The percentage reduction in alamarBlue was calculated using the given formula.

$$\% \text{ Reduced} = \frac{(\varepsilon ox\ \lambda 2)\,(A\ \lambda 1) - (\varepsilon ox\ \lambda 1)\,(A\ \lambda 2)}{(\varepsilon red\ \lambda 1)\,(A'\ \lambda 2) - (\varepsilon red\ \lambda 2)\,(A'\ \lambda 1)} \times 100\ \%$$

($\varepsilon red\ \lambda 1$) = 155,677 (Molar extinction coefficient of reduced alamarBlue at 570 nm)

($\varepsilon red\ \lambda 2$) = 14,652 (Molar extinction coefficient of reduced alamarBlue at 600 nm)

($\varepsilon ox\ \lambda 1$) = 80,586 (Molar extinction coefficient of oxidized alamarBlue at 570 nm)

($\varepsilon ox\ \lambda 2$) = 117,216 (Molar extinction coefficient of oxidized alamarBlue at 600 nm)

($A\ \lambda 1$) = Absorbance of test wells at 570 nm

($A\ \lambda 2$) = Absorbance of test wells at 600 nm

($A'\lambda 1$) = Absorbance of negative control wells which contain medium plus alamarBlue but to which no cells have been added at 570 nm.

($A'\lambda 2$) = Absorbance of negative control wells which contain medium plus alamarBlue but to which no cells have been added at 600 nm.

The cell proliferation rate was assessed between the study groups; HGF-1 without *C. nutans* treatment (*Control)*, HGF-1 treated with 250 µg/ml concentration of

ethanolic leaf extract of *C. nutans* (*Group 1)*, HGF-1 treated with 125 µg/ml concentration of ethanolic leaf extract of *C. nutans* (*Group 2),* HGF-1 treated with 62.5 µg/ml concentration of ethanolic leaf extract of *C. nutans (Group 3),* and the negative control (culture medium with no cells) in order to detect the difference in reduction percentage using alamarBlue.

The data were analysed using SPSS 24 for windows and Kruskal-Wallis test to compare the proliferative activity of HGF-1 cells which were subjected to treatment with 250, 125, and 62.5 µg/ml of ethanolic extracts of *C. nutans* leaves and control.

Results

Based on Kruskal-Wallis H test, there was statistically a significant difference in the reduction percentage of alamarBlue between the proliferative activity of HGF-1 cells treated with different concentrations of ethanolic leaf extracts of *C. nutans* and control; $\chi^2(3) = 12.678$, $p = 0.005$, with a mean rank score of 9.29 for 250 µg/ml, 10.29 for 125 µg/ml, 23.29 for 62.5 µg/ml and 15.14 for the control. A post hoc analysis showed that the mean ranks for the control and 62.5 µg/ml were 4.71 and 10.29; there was a significant difference in the distributions between the two groups (Mann-Whitney U = 5, n1 = n2 = 7, p=0.011 two tailed). The mean ranks between the control with the 250 µg/ml and control with the 125 µg/ml was insignificant, p=0.128, and p=0.165 respectively, when tested with the Mann-Whitney U test. This shows that there was a significant increase in cell proliferation in HGF-1 when treated with 62.5 µg/ml concentration of ethanolic extract of *C. nutans* leaves. The mean absorbance values of alamarBlue at 570 nm and 600 nm wave length for the tested groups are shown in Table 1. The percentage of alamarBlue reduction compared between the tested groups was significant (p=0.005) as shown in Table 2.

Table 1 Absorbance values of alamarBlue assay measured spectrophotometrically at 570nm and 600nm of human gingival fibroblasts treated with *Clinacanthus nutans*

Day	Control		Group 1		Group 2		Group 3		Negative control	
	570	600	570	600	570	600	570	600	570	600
1	1.010	0.193	1.007	0.184	0.999	0.188	1.048	0.196	0.702	0.614
2	0.944	0.181	0.967	0.172	0.934	0.172	1.010	0.191	0.693	0.606
3	0.941	0.180	0.944	0.189	0.918	0.170	0.952	0.178	0.665	0.586
4	0.968	0.178	0.980	0.203	0.952	0.172	0.992	0.180	0.617	0.589
5	0.995	0.189	0.962	0.215	0.964	0.180	1.031	0.190	0.687	0.613
6	0.981	0.200	0.956	0.262	0.928	0.177	1.014	0.191	0.688	0.617
7	0.924	0.203	0.865	0.303	0.892	0.202	0.926	0.191	0.636	0.580

Table 2 Percentage of alamarBlue reduction between the control and tested groups

Variable	Groups	*N*	Mean rank	χ^2 statistic (df)[a]	*p* value[a]
alamarBlue reduction %	Control	7	15.14	12.678 (3)	0.005
	Group 1	7	9.29		
	Group 2	7	10.29		
	Group 3	7	23.29		

[a] Kruskal-Wallis test, $p < 0.05$

Discussion

C. nutans plant has been traditionally used in the treatment of *Herpes* infection, diabetes, cancer, inflammation as well as various skin problems (Alam *et al.,* 2016). *C. nutans* also displayed anti-inflammatory, antiedemic, antipyretic, analgesic as well as menstrual regulation activities (Satayavivad *et al.,* 1996). The young leaves of this plant are consumed in Malaysia for the maintenance of health and its fresh leaves are commonly eaten as herbal tea to treat diabetes, fever, skin rashes as well as in the form of diuretics (Shim *et al.,* 2013). *C. nutans* has been claimed to have traditional therapeutic use against *Herpes simplex* virus infection, the viral lesions in vesicular stomatitis as well as to relieve minor skin inflammation and insect bites (Charuwichitratana *et al.,* 1996). The fresh leaves of this plant have been used traditionally in Thailand as an anti-inflammatory drug in treating snake and insect bites, skin rashes, allergies, the lesions in the case of *Herpes simplex* and *Varicella-zoster* infection (Sakdarat *et al.,* 2006). *C. nutans* leaves are consumed traditionally as herbal tea alone or as a medicine by cancer patients in Malaysia, Thailand, and Singapore with also claims made that consumption of this at high doses has cured cancer (Poonthananiwatkul *et al.,* 2015). Despite many research conducted on the therapeutic effect of *C. nutans* in treating various diseases, there is still dearth of information on the proliferative activity of HGF-1 treated with *C. nutans.* HGFs are one of the types of cells that have been used in cell cultures owing to its ease of culture following the enzymatic digestion method described by Supraja *et al.* (2016). Also, Giannopoulou and Cimasoni (1996) have reported that the establishment of primary gingival fibroblast culture is simple, as these cells adhere to the culture plates and spread well enabling good proliferation without the requirement of specific culture conditions. Gingival connective tissue comprises HGFs as its main cell population which are involved in the production of extracellular matrix of tissue, both in disease and health (Bartold *et al.*, 2000; Poggi *et al.*, 2003). HGF cells are the most abundant cell type that make up the periodontal connective tissues in mouth (Lee *et al.*, 2013). Moreover, HGFs play a vital role in wound healing in the oral cavity and are functionally and phenotypically different from the skin fibroblasts (Stephens *et al*., 2001; Sukotjo *et al*., 2003; Lin *et al*., 2010). Hence, this led to the choice of HGF-1 in the current study.

There are many ways in which cell proliferation can be measured. One is by measuring the metabolic activity of the population cells. AlamarBlue assay is intended to measure the proliferation of several human and animal cell lines. This alamarBlue assay is simple to be performed due to its water solubility, culture medium stability, non-toxicity and its ability in not altering the cell viability cultured many times (Ahmed *et al.,* 1994). In this study, the assessment of proliferation throws light on the cytotoxic effect of the ethanolic extract of leaves of *C. nutans* on HGF-1 cell line. One *in-vitro* study (Sriwanthana *et al*., 1996), suggested that ethanolic extract of *C. nutans* leaves at 0.5, 2.5, and 5.0 µg/ml, promoted lymphocyte proliferation in human competent cells but decreased drastically at concentrations of 2.5 and 5.0 mg/ml of extract. The results found in this study are in agreement with the previous research which represents an association between low concentrations of ethanolic leaf extract of *C. nutans* with the increase in proliferation in normal human cell line. Another study by P'ng *et al*. (2013) suggested that the methanolic extract of *C. nutans* leaves at concentrations of 300, 600, and 900 mg/kg, exhibited no toxicological effect on the kidney and liver of Sprague Dawley female rats through oral administration for 14 days.

There were many studies conducted regarding different crude extracts of *C. nutans* leaves on different human cancer cell lines, but few have been tested with the ethanolic crude leaf extracts of *C. nutans*. Yong *et al.* (2013) found divergent effectiveness in chloroform, aqueous and methanol crude leaf extracts on various cancer cell lines from human, namely, human liver hepatocellular carcinoma (HepG2), human lung cancer cell line (NCI-H23), human neuroblastoma cell line (IMR-32), human gastric cancer cell line (SNU-1), human erythroleukemia cell line (K-562), human colon adenocarcinoma cell line (LS-

174T), human Burkitt's lymphoma cell line (Raji) as well as human cervical cancer cell line (HeLa). They showed that chloroform extract of this leaf had the highest anti-proliferative effect on K-562 and Raji cell lines. In yet another study by Arullappan *et al.* (2014), it was shown that the *in vitro* cytotoxicity study conducted on petroleum ether, methanol and ethyl acetate extracts of the leaves from *C. nutans* based on MTT assay, there was inhibition on HeLa with K-562 with IC_{50} values of 18 and 20 µg/ml respectively in the case of petroleum ether extract. Moreover, there are also limited studies done in determining the cytotoxicity of ethanolic extracts of *C. nutans* leaves on normal human cell lines as compared to human cancer cell lines.

Several researches suggest that the ecological factors affecting plant growth condition can alter metabolic pathway causing a change in total concentration of bioactive compounds ultimately affecting the amount of bioactivities present (Ramakrishna *et al.,* 2011; Radušienė *et al.,* 2012). A study based on the crude methanol extracts from the leaves of *Justicia gendarussa* belonging to the family Acanthaceae, which were obtained from different locations in Malaysia showed different levels of cytotoxicity on MDA-MB-231 and -468 breast cancer cell lines (Ayob *et al.,* 2014). The bioactive components in the *C. nutans* leaves used in the current study may vary in comparison to other researches which could be due to environmental and ecological factors attributing to equivocal results. However, to ascertain compounds responsible for the properties of crude extracts of *C. nutans* leaves, bioassay-guided fractionation, purification as well as isolation of the bioactive compounds should be carried out.

In the current study, a significant increase in cell proliferation of HGF-1 was observed on treatment with a concentration of 62.5 µg/ml of ethanolic extract of *C. nutans* leaves. It has been reported previously that through the process of efficient reprogramming of gingival fibroblasts, it is possible to make the gingiva a good source for induced pluripotent stem (iPS) cells which could be used for drug screening applications and for autologous cell therapy in the field of dentistry (Egusa *et al*., 2010). Hence, the results of this study could be exploited in tissue engineering and regenerative medicine as well as in clinical applications such as in oral wound healing in future.

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