Research article

Phytochemical analysis and cytotoxicity of *Clinacanthus nutans* on human gingival fibroblast cell line

A'attiyyah Ab Alim[1], Wan Afiqah Syahirah Wan Ghazali[1], Nor Azah Mohd Ali[2], Kannan Thirumulu Ponnuraj[1,3], Suharni Mohamad[1], Ahmad Azlina[1]

[1]School of Dental Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia
[2]Herbal Product Development Laboratory, Natural Products Division, Forest Research Institute of Malaysia, Kepong, 52109 Selangor, Malaysia
[3]Human Genome Centre, School of Medical Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia

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Corresponding author: **Kannan Thirumulu Ponnuraj.** Email: kannan@usm.my

ABSTRACT

Introduction and Aim: *Clinacanthus nutans* (*C. nutans*) possesses antimicrobial, anticancer, and anti-inflammatory activities. The present study aimed to carry out phytochemical analyses of aqueous and ethanolic extracts of *C. nutans* leaves and also its cytotoxic potential on human gingival fibroblast cell line HGF-1.

Materials and Methods: Phytochemical analyses was conducted using Gas Chromatography Mass Spectrometry (GCMS). Cytotoxicity testing was carried out by treating the HGF-1 with different concentrations (50,100, 200, 400, 800, 1600, 3200, 6400 µg/ml) of both the aqueous and ethanolic extracts of *C. nutans* leaves using MTT assay. The control comprised only the cell suspension and culture medium.

Results: Phytochemical study revealed 53 compounds. Furfural, 2-furanmethanol, butyrolactone, phenol, benzeneacetaldehyde, 4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl-, vitamin E, campesterol, stigmasterol,.gamma.-Sitosterol and lupeol were the compounds identified in both extracts. Both the extracts did not exhibit any cytotoxicity on HGF-1 based on MTT assay with IC_{50} values of 1099 µg/ml (ethanol extract) and 2627 µg/ml (aqueous extract).

Conclusion: This study showed that ethanol and aqueous extracts of *C. nutans* leaves contained varied phytochemical compounds and were non-cytotoxic on human gingival fibroblast cell line under the present test conditions.

Keywords: *Clinacanthus nutans;* ethanol; aqueous; cytotoxicity; human gingival fibroblasts.

INTRODUCTION

Clinacanthus nutans (*C. nutans*) or Sabah snake grass is a small shrub belonging to family of Acanthacea and is native to the tropical regions of Southeast Asia, primarily Malaysia, Thailand, Indonesia, southern China, and some temperate regions (1) and in Malaysia, *C. nutans* is called Belalai Gajah (2). Previous studies on *C. nutans* have reported that this plant possesses antioxidant (3), anti-proliferative (4), and antimicrobial activities (5). It has been reported on the isolation of various phytochemical compounds such as stigmasterol, lupeol, β-sitosterol, botulin (6), and six known C-glycosyl flavones (vitexin, isovitexin, shaftoside, isomollupetin, 7-O-β-glucopyranoside, orientin and isorientin) isolated from n-BuOH and water-soluble fractions of the methanolic extract of *C. nutans* (7). Even though *C. nutans* has been used traditionally by folklores to treat various diseases internally and externally, the possibility of *C. nutans* to be cytotoxic towards consumers cannot be ignored. The facts that medicinal plants contain advantageous bioactive compounds are known widely; however, the facts that these bioactive compounds might be poisonous and contain toxic compounds are not really exposed to the consumers. Hence, the aim of this study was to determine the phytochemical compounds present in the leaves of *C. nutans* using Gas Chromatography Mass Spectrometry (GCMS) and to study its cytotoxicity using MTT assay on human gingival fibroblast (HGF-1) cell line.

MATERIALS AND METHODS

Collection of plant material

Pesticide free leaves of Sabah snake grass (*C. nutans*) were collected from TKC Herbal Nursery Sdn. Bhd., Seremban, Negeri Sembilan, Malaysia. The specimen was sent to Bioresource Unit under Herbal Product Development Programme, Forest Research Institute of Malaysia (FRIM) for further identification and authentication. A voucher specimen (PPH17001) was identified for *C. nutans* at FRIM. The fresh leaves of *C. nutans* were cut into small pieces and oven-dried at 40-45°C for 1-2 days until constant dry weight. Then the leaves were mechanically ground using heavy duty blender into fine powder and placed in clean paper bag with proper labelling.

Preparation of extracts from *Clinacanthus nutans* leaves

The leaves of *C. nutans* were soaked in solvents (water and ethanol, with the ratio of one part of powder to ten parts of solvent). The aqueous extractions were carried out on dried leaf under reflux for 8 hours, while ethanolic extraction was performed using maceration technique on the dried leaves of *C. nutans*. Samples of the leaves were soaked in 70% of ethanol for 72 hours. At the end of each respective extraction, the filtrates were obtained by filtering the crude extracts using Whatman filter paper no.1 (Whatman Ltd., England). Next, under pressure at 40°C, using a rotary evaporator, the solvent was evaporated to obtain the extract. The aqueous extract was frozen, placed in a freeze-dry machine for 3-4 days. The ethanol extract was rotary-dried, and both the extracts were weighed, and the percentage of each extract was determined. The extracts were stored in freezer at 4°C until further analysis.

Gas chromatography-mass spectrometry analysis

For GCMS analysis, Agilent Technologies Gas Chromatography Mass Spectra (Hewlett Packard 6890 series Gas Chromatograph with 5973N Mass Selective Detector and Chemstation Data System) was used to identify the chemical compounds present in the ethanol extracts. The temperature of the experiment was programmed as detailed below: the initial temperature was set at 50°C for 2 minutes, with an increase of 20°C/min to 280°C, hold for 10 minutes. The compounds were identified based on the comparison of their mass spectra with standards and with the library of National Institute Standard and Technology (NIST). Further confirmation of the identity of the chromatographic peaks was done by spiking using reference standards.

Cytotoxicity assay

Cell culture

HGF-1 (human gingival fibroblasts) (ATCC®CRL-2014™) cell line was purchased from American Type Culture Collection (ATCC, USA). HGF-1 were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 4 mM L-glutamine, 4500 mg/l glucose, 1 mM sodium pyruvate, and 1500 mg/l sodium bicarbonate and supplemented with 10 per cent Foetal Bovine Serum (FBS) and 1 % penicillin-streptomycin solution. The cells were cultured in an incubator at an atmosphere of 5 % CO_2 and 95 % air at 37°C.

In vitro assay for cytotoxic activity

The ethanol and aqueous extracts of *C. nutans* leaves were tested for *in vitro* cytotoxicity on HGF-1 using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, the cultured HGF-1 were washed using phosphate buffered saline (PBS) and harvested by trypsinization. Then, the cells were plated at a density of 1×10^4 cells/ml in 96-well microtiter plate and incubated under 5 % CO_2 and 95 % air at 37°C for 24 hours. For treatment groups, the HGF-1 were treated with different concentrations of plant extracts including 50,100, 200, 400, 800, 1600, 3200, 6400 µg/ml. In control well, only cell suspension and culture medium were used. All concentrations of extracts were carried out in triplicates on the same cell batch. Each sample was replicated three times and the cells were grown in an incubator at 37°C and 5 % CO_2 for 72 hours. After incubation, MTT solution (10 µl of 5 mg/ml in PBS) was incorporated into each well following which the plates were subjected to incubation for another 4 hours until purple precipitates were clearly visible under a microscope. The medium as well as the MTT solution were removed from the wells and the formazan crystals produced by viable cells were dissolved in 100 µl DMSO with gentle shaking of the plates. The absorbance was measured at a wavelength of 570 nm using ELISA plate reader in a microtiter plate reader and the percentage cell viability (CV) was calculated. The effects of extracts were expressed by IC_{50} values.

RESULTS

Percentage yield of crude extracts of *Clinacanthus nutans* leaves

After the evaporation of the extract's solvent by rotary evaporator and freeze drying, the weight of crude extracts of the leaves of *C. nutans* obtained was determined. The weight of dried fine powder was 500 g and the weight after solvent evaporation was 56.10 g for the ethanol extract and 72.0 g for the aqueous extract. The percentage yield of aqueous extract was higher (14.40%) than the percentage yield of ethanol extract (11.22%).

Chemical composition of extracts by GCMS analysis

Forty-four compounds of ethanol extract were identified and characterized using GCMS analysis (Table 1). These compounds can be classified as phenolic compounds, flavonoids, terpenoids, esters, fatty acids, ketones, furans, sulphur, nitrogen compounds and hydrocarbons. 2-Furancarboxaldehyde, 5-(hydroxymethyl)- (5.86%) and n-Hexadecanoic acid (2.55%) were identified as the main chemical compounds in ethanol extract of *C. nutans* leaves. Meanwhile, only twenty-nine compounds could be identified and characterized (Table 1) by GCMS analysis. These compounds comprised phenolic compounds, terpenoids, esters, fatty acids, ketones, furans, sulphur, and nitrogen compounds groups. The major top two chemical compounds identified were 4H-Pyran-4-one, 2, 3-dihydro-3, 5-dihydroxy-6-methyl- (6.91%) and Benzofuran, 2, 3-dihydro- (3.95%).

Table 1: Chemical compounds of ethanol and aqueous extracts of leaves of *C. nutans*, groups and their retention time

Sl.No	Compounds	Ethanol		Aqueous	
		RT	Area %	RT	Area %
	Phenolic compounds				
1	Phenol	7.009	0.27	7.002	0.33
2	Benzeneacetaldehyde	7.681	0.35	7.674	1.15
3	Phenol, 2-methoxy-	-	-	8.116	0.24
4	4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl-	8.697	2.41	8.627	6.91
5	Benzoic acid	8.809	0.4	8.76	1.58
6	Benzofuran,2,3-dihydro-	-	-	9.082	3.95
7	2-Methoxy-4-vinylphenol	9.712	0.34	-	-
8	Phenol,2,6-dimethoxy-	9.929	0.86	9.922	1.39
9	Phenol,2,4-bis(1,1-dimethylethyl)-	10.762	2.23	-	-
10	4-(1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol	-	-	11.911	1.22
	Flavonoids				
11	Pentaethylene glycol monododecyl ether	14.144	0.45	-	-
12	Hexaethylene glycol monododecyl ether	15.02	0.28	-	-
	Terpenoids- Triterpenoids/steroids				
13	Phytol	13.325	0.56	13.318	0.23
14	Squalene	15.727	1.33	-	-
15	.gamma.-Tocopherol	16.763	0.24	-	-
16	Vitamin E	17.253	2.62	17.218	0.06
17	Campesterol	17.995	0.24	17.988	0.09
18	Stigmasterol	18.226	0.57	18.212	0.18
19	.gamma.-Sitosterol	18.689	0.79	18.661	0.34
20	.beta.-Amyrin	19.081	0.13	-	-
21	Lupeol	19.466	0.16	19.459	1.07
	Carotenoids				
22	Megastigmastrienone	11.385	0.29	-	-
	Fatty acids				
23	Tetradecanoic acid	11.883	3.33	-	-
24	n-Hexadecanoic acid	12.751	2.55	12.716	1.11
25	9,12-Octadecadienoic acid, (Z,Z)-	-	-	13.395	0.52
26	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	13.437	1.14	-	-
27	Octadecanoic acid	13.507	1.71	13.479	0.63
	Alkanes and Hydrocarbons				
28	1-Undecanol	10.006	0.41	-	-
29	Cyclododecane	10.545	0.24	-	-
	Esters				
30	Hexadecanoic acid, methyl ester	12.576	0.37	12.576	0.44
31	Hexadecanoic acid, ethyl ester	12.849	0.32	12.842	0.32
32	7,10,13-hexadecatrienoic acid, methyl ester	13.276	0.48	-	-
33	Ethyl 9,12,15-octadecatrienoate	13.528	0.68	-	-
34	Octadecanoic acid, ethyl ester	13.591	0.26	-	-
35	Hexadecanoic acid, 2,3-dihydroxypropyl ester	14.683	0.31	-	-
36	Methyl 9,12-heptadecadienoate	15.258	0.27	-	-
	Ketones				
37	Butyrolactone	5.924	0.13	5.938	1.42

38	2-Cyclopenten-1-one, 2-hydroxy-	6.162	0.45	-	-
39	2-Cyclopenten-1-one, 2-hydroxy-3-methyl-	-	-	7.527	0.24
40	1,2-Cyclopentanedione, 3-methyl-	7.541	0.27	-	-
	Furans				
41	Furfural	3.872	0.35	3.767	0.19
42	2-Furanmethanol	4.503	0.39	4.454	0.49
43	2(5H)-Furanone	5.959	0.13	-	-
44	3-Furanmethanol	-	-	6.169	0.91
45	2-Furancarboxaldehyde, 5-methyl-	6.736	0.21	-	-
46	2,5-Dimethyl-4-hydroxy-3(2H)-furanone	8.011	0.3	-	-
47	2-Furancarboxaldehyde,5-(hydroxymethyl)-	9.334	5.86	-	-
	Sulphurs				
48	Dimethyl trisulfide	6.764	0.73	6.757	1.16
49	S-Methyl methanethiosulphonate	7.934	2.23	7.906	2.46
	Nitrogen compounds				
50	Pyrazine, methyl-	-	-	3.578	0.18
51	Indole	-	-	9.6	0.8
52	L-Proline,5-oxo-,methyl ester	-	-	10.097	0.55
53	7-Azaindole-3-carboxaldehyde	10.468	0.65	-	-

******RT = Retention time

Cytotoxicity effect of *Clinacanthus nutans* extracts

HGF-1 were incubated with various concentrations of ethanol and aqueous extracts of *C. nutans* leaves for 72 hours and then assayed by MTT assay to determine the cytotoxicity. Results of different concentrations of ethanol and aqueous extracts of leaves of *C. nutans* are expressed as a mean ± standard deviation (SD) in Table 2.

Table 2: Cell viability of HGF-1 treated with ethanol and aqueous extracts of *C. nutans* leaves by MTT assay

Concentration (µg/ml)	Cell viability of HGF-1 (%)	
	Ethanol extract	Aqueous extract
6400	13.8 ± 1.70	46.31 ± 9.25
3200	42.23 ± 5.83	60.14 ± 6.54
1600	50.08 ± 5.13	60.5 ± 8.35
800	56.88 ± 5.15	68.95 ± 7.31
400	66.04 ± 7.39	63.57 ± 7.58
200	71.07 ± 6.20	69.84 ± 4.06
100	77.98 ± 3.05	71.1 ± 7.36
50	80.27 ± 1.10	75.98 ± 1.29
25	87.98 ± 3.44	95.12 ± 2.26
Control	100.00 ± 0.0	100.00 ± 0.0

MTT assay of ethanol extract of *C. nutans* leaves showed significant effect on HGF-1 in concentrations ranging between 3200 to 25 µg/ml when compared with the control (cells with culture medium only). However, the aqueous extract of *C. nutans* leaves showed significant effect on HGF-1 at all concentrations except at 25 µg/ml concentration with 95.12 ± 2.26 % of cell viability. Statistical analysis based on general linear model (GLM) using Statistical Package for the Social Sciences (SPSS) statistics version 20 showed that higher the concentrations of the *C. nutans* leaf extracts, lower was the cell viability for both the ethanol and aqueous extracts of leaves of *C. nutans*. According to pairwise comparison in SPSS analysis, the cell viability of ethanol and aqueous extracts of leaves of *C. nutans* showed significant difference among each other with value of $p < 0.01$. GraphPad Prism software version 5 was used to perform non-linear regression analysis to determine the IC_{50} value for ethanol and aqueous extracts of leaves of *C. nutans* on HGF-1. IC_{50} values for ethanol and aqueous extracts of leaves of *C. nutans* were 1099 µg/ml and 2627 µg/ml respectively with a 95% confidence interval of 623.8 to 1935 for the ethanol extract and 1025 to 6727 for the aqueous extract.

DISCUSSION

Most of the medicinal plant extracts are not well documented for their toxicity effects because of the widely held belief that medicinal plants are safe (8). Zhang and team in their study suggested the use of ethanol or water or the mixtures of ethanol and water to prepare extracts as the polarity difference would enable extracting different compounds and due to their suitability for human consumption (9). The differences in percentage yield of ethanol and aqueous extracts of *C. nutans* leaves could be due to the effectiveness of extracting techniques regardless of the plant material and solvent used (10). Extracting plants by hot solvent systems under reflux give higher percentage yield. Polarity of the solvents used is very important in determining the types and percentage of compounds that will be extracted. Do et al. reported

that ethanol was a good solvent for the extraction of polyphenols and safe to be consumed by humans (11). Several groups of phytochemical compounds were detected in ethanol and aqueous extracts of *C. nutans* with most groups being detected in ethanol extract was fatty acids, while, in aqueous extract was phenols. Other groups were also identified in the extracts of *C. nutans* including flavonoids, terpenoids, alkanes and hydrocarbons, esters, furans, ketones, sulfurs, and nitrogen compounds, with flavonoids and alkanes and hydrocarbons groups were not detected in aqueous extract. Overall, 53 phytochemical compounds (Table 1) were detected in ethanol and aqueous extracts of *C. nutans*.

Phenolic compounds derived from the secondary metabolism of plants are divided into 2 groups which are flavonoid and non-flavonoid polyphenols. Flavonoid compounds were detected in ethanol extract only and not in the aqueous extract. This could be due to the different polarity of the solvents employed for extraction. The recovery of phenolic compounds depended on solvents used, the solubility and polarity of phenolic compounds in the solvents used for extraction (12).

Phytol ($C_{20}H_{40}O$) is an example of diterpenes (C20), meanwhile, triterpenes (C30) are steroids, sterols, and cardiac glycosides (13). Squalene ($C_{30}H_{50}$), beta-amyrin ($C_{30}H_{50}O$) and lupeol ($C_{30}H_{50}O$) are triterpenes found in *C. nutans* extracts leaf. Both extracts of *C. nutans* leaves were found to contain phytol and this compound is known as a reactive oxygen species-promoting substance (14). Phytol also exhibited anti-inflammatory and antioxidant effects and was proven to give relief to patients suffering from chronic inflammatory disorders of airways (asthma) (14). Study by Jain and her colleagues showed that vitamin E was found to suppress the increase in airway reactivity in guinea pigs sensitized to ovalbumin (15). Vitamin E is a well-known antioxidant that might be helpful to combat cancers including oral cancer. In 2014, Iqubal and colleagues reviewed on vitamin E and its role in prevention of oral cancer (16).

McGaw in his review reported on the antibacterial activities of fatty acids (17). Saturated fatty acids are effective against microorganisms at lower chain length, whereas monounsaturated and polyunsaturated fatty acids are effective against microorganisms at longer chain lengths. Regarding ester groups, ethyl ester, hexadecanoic acid possess hypocholesterolemic and antimicrobial properties while hexadecanoic acid, methyl ester is known for its antibacterial (18) and antifungal activities (19). Other ester groups such as methyl 9,12-heptadecadienoate has anticancer properties (20). Ketone groups such as butyrolactone exhibited strong free radical scavenging activity and can increase the total phenolic contents in plants (21). Overall, the chemical groups found in *C. nutans* leaves extracts play important roles on the pharmacological properties exhibited by phytochemical compounds. Based on the present study, *C. nutans* leaves do not have any phytochemical compounds in alkaloid groups.

MTT assay of ethanol extract of *C. nutans* leaves showed significant effect on HGF-1 in concentration range between 3200 µg/ml to 25 µg/ml when compared with the control (cells with culture medium only). However, the aqueous extract of *C. nutans* leaves showed significant effect on HGF-1 at all concentrations except at 25 µg/ml concentration with 95.12 ± 2.26 % of cell viability. According to Behzad and his colleagues if the IC_{50} > 100 µg/ml in MTT assay, the plant extract was considered inactive and non-toxic to the cells (22). The cytotoxicity activity of plant extracts is classified into three categories: potentially cytotoxic (IC_{50} < 100 µg/ml), moderately cytotoxic (100 µg/ml < IC_{50} < 1000 µg/ml) and non-cytotoxic (IC_{50} > 1000 µg/ml). In this study, the ethanol, and aqueous extracts of leaves of *C. nutans* can be classified as non-cytotoxic since their IC_{50} > 1000 µg/ml (23). Recent study on the cytotoxicity of methanol extract of *C. nutans* using MTT assay against human liver cancer cell line (Hep-G2), human lung carcinoma cell line (A549), human colorectal adenocarcinoma cell line (HT-29), human breast adenocarcinoma cell line (MDA-MB-231), human breast adenocarcinoma cell line (MCF-7), human gastric adenocarcinoma cell line (CRL 1739) and normal cells (3T3) showed that *C. nutans* exhibited cytotoxic effects on Hep-G2 (IC_{50} = 13.33 µg/ml) and MDA-MB-231 (IC_{50} of 18.67 µg/ml) (24). Based on this study, *C. nutans* was found to be non-cytotoxic to normal cells, 3T3, and cytotoxic against certain cancer cells. Fong and team studied the cytotoxic effects of methanol extract of *C. nutans* against D24 melanoma and normal human dermal fibroblasts (NHDF) and the results showed significant cytotoxicity only on D24 melanoma cells (36). The present study also showed that ethanol and aqueous extracts of leaves of *C. nutans* did not exhibit cytotoxic effects on HGF-1. Hence, results of the present study agree with previous finding that extract of *C. nutans* was selective against cancer cells but not normal cells (25). *C. nutans* leaves of ethanol and aqueous extracts possess anticancer properties but are only limited to cancer cells and does not affect normal cells.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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