Pharmacogn J. 2021; 13(4): 1014-1018
A Multifaceted Journal in the field of Natural Products and Pharmacognosy
www.phcogj.com

Research Article

Cytotoxic Activity of *Eurycoma Longifolia* Jack Root Extract Against Nasopharyngeal Carcinoma Cell Line

Nur Haseena Kajahmohideen[1], Siti NurSyafiqah Razi[1], Ghasak Ghazi Faisal[2,*], Abdelkader Elbadawy Ashour[3], Anisa Kusumawardani[4], Essam A. Makky[5], O.E. Ibrahim[6]

ABSTRACT

Background: *Eurycoma longifolia (E. longifolia)* or Tongkat Ali is a tree that grows in southeast Asia, the roots of which contain bioactive components that exhibit cytotoxic properties against various cancer cell lines. However, no study has been conducted to relate the cytotoxic properties against nasopharyngeal carcinoma (NPC), a type of cancer that shows poor prognosis for metastatic disease. The purpose of this study was to determine whether the *E. longifolia* root extract exerts cytotoxic activity against nasopharyngeal carcinoma (ORL-115) cell lines. **Materials and Method:** *E. longifolia* root extracts were obtained through Soxhlet extraction method and by using two different solvents; ethanol and dicholoromethane. MTS assay was used to evaluate the cytotoxic effect of the root extracts against ORL-115 cell line for three different incubation time which were 24-hour, 48-hour and 72-hour. **Results:** Ethanol extract was significantly more potent compared to DCM extract. Ethanol extract exhibited lower IC_{50} value compared to DCM extract. The IC_{50} of ethanol extract were 232.1 µg/ml, 66.86 µg/ml and 42.6 µg/ml. Meanwhile the IC_{50} of DCM extract were 678.87 µg/ml, 136.71 µg/ml, 73.72 µg/ml for 24-hour, 48-hour and 72-hour incubation period respectively. The cytotoxic activity of both extracts increased as the incubation time prolonged. The cytotoxic activity of ethanol extract at each incubation time was significantly different from DCM extract except at 72 hours. **Conclusion:** *E. longifolia* root extracts exerted cytotoxic activity against the nasopharyngeal carcinoma (ORL-115) cell line. Ethanol extract exhibited lower IC_{50} value compared to DCM extract. The cytotoxic activity of both extracts were dose dependent and time dependent.
Key words: *E. longifolia,* Cytotoxic activity, Nasopharyngeal carcinoma.

Nur Haseena Kajahmohideen[1], Siti NurSyafiqah Razi[1], Ghasak Ghazi Faisal[2,*], Abdelkader Elbadawy Ashour[3], Anisa Kusumawardani[4], Essam A. Makky[5], O.E. Ibrahim[6]

[1]Kulliyyah of Dentistry, IIUM, MALAYSIA.
[2]Lecturer, Department of fundamental dental and medical sciences, Kulliyyah of Dentistry, IIUM, MALAYSIA.
[3]Lecturer, Basic Medical Science Unit, Kulliyyah of Medicine, IIUM, MALAYSIA.
[4]Department of Restorative Dentistry, Kuliyyah of Dentistry, IIUM, MALAYSIA.
[5]Faculty of Industrial Sciences & Technology, Universiti Malaysia Pahang (UMP), Gambang, 26300 Kuantan, MALAYSIA
[6]Faculty of Dentistry, Universiti Teknologi MARA, MALAYSIA.

Correspondence

Ghasak Ghazi Faisal

Lecturer, Department of fundamental dental and medical sciences, Kulliyyah of Dentistry, IIUM, MALAYSIA.

E-mail: drghassak@yahoo.com

History

- Submission Date: 06-04-2021;
- Review completed: 28-05-2021;
- Accepted Date: 08-06-2021.

DOI : 10.5530/pj.2021.13.131

Article Available online

http://www.phcogj.com/v13/i4

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a cancer that originates from the epithelium of the nasopharynx. The carcinoma appears to be frequently arise from fossa of Rosenmüller (FOR) which is located within the boundaries of the nasopharynx. From this site, the carcinoma can invade adjacent anatomical spaces or organs [1]. According to Petersson [2], the anatomy of FOR may prevent early detection of NPC due to the fact that it is often too deep and narrow for clinical-endoscopic examination. Regarding the survival rate of the patients, he mentioned that according to American Cancer Society, the 5-year survival rate for patients with stage III, IVa and IVb drops below than 50% to around 30-40%. The 5-year survival rate for patients with stage IVc was poorer which is only less than 10%.

The treatment modalities for NPC are based on the stages of the disease. Radiotherapy alone is indicated in stage I disease. Stage II disease is to be treated with radiotherapy with or without concurrent chemotherapy and stage III to stage IVb is treated with radiotherapy and concurrent chemotherapy. Despite the achievement of excellent local control, distant failure remains a problem and there is still poor treatment outcome for metastatic disease [3]. Long term survivors of NPC may face several complications due to the effects of radiotherapy on organs adjacent to nasopharynx such as xerostomia, carotid artery stenosis and osteoradionecrosis. Ototoxicity can result from the use of cisplatin in chemotherapy. There is also report on the development of secondary malignant tumor such as osteosarcoma, adenocarcinoma and squamous cell carcinoma after NPC treatment [2].

Recently, a lot of natural products such as plants have been identified to act as anti-cancer agents due to the fact that they have abundant numbers of pharmacologically active compounds that are safe to the body [4]. Researches are being conducted to identify the bioactive components that can exert the cytotoxic effect against various cancer cell-lines.

Eurycoma longifolia (E.longifolia) Jack or also known as Tongkat Ali is a type of herbal plants from Simaroubaceae family and are largely found in Southeast Asia [5]. This plant has been traditionally used for its aphrodisiac effect as well as for the treatment of malaria and persistent fever [6]. Another beneficial property of *E. longifolia* is its cytotoxic activity towards cancer cells. The cytotoxic activity was attributed to the presence of numerous bioactive constituents such as eurycomalactone, longilactone, pasakbumin B, eurycomanone and β-carboline alkaloids [7].

Mohamed, Vejayan and Yusoff [4] briefly described a few studies that discovered the mode of action of *E. longifolia* that contributed to the death of various cancer cell lines. One of the bioactive components of *E. longifolia* is *eurycomanone* that causes the down-regulation of the anti –apoptotic protein Bcl-2, up-regulation of p53 tumor suppressor protein and elevation of Bax protein which is a type of pro-apoptotic protein. These actions were said to be responsible for the anti-proliferative effect of

Cite this article: Kajahmohideen NH, Razi SN, Faisal GG, Ashour AE, Kusumawardani A, Makky EA, et al. Cytotoxic Activity of Eurycoma Longifolia Jack Root Extract Against Nasopharyngeal Carcinoma Cell Line. Pharmacogn J. 2021; 13(4): 1014-1018.

the plant extract. Aside from that, he stated that the phenolics and flavonoid contents of *E. longifolia* showed anti-tumor effect through the free radicals scavenging activity, hence preventing the detrimental effects of the free radicals towards DNA that can lead to cancer.

In their article, Rehman, Choe and You [7] mentioned the previous researches that had discovered positive cytotoxic activity of *E. longifolia* against various cancer cell lines such as human lung cancer (A-549), human breast cancer (MCF-7), human papillomavirus (KB), human prostate cancer (DU-145), human rhabdomyosarcoma (RD) and ovarian cancer (CaOV-3) cell lines. Interestingly, in a study done by Nurhanan *et al.* [8] there is no significant cytotoxicity of the root extracts of *E. longifolia* against MDBK (kidney) normal cell line compared to tamoxifen, an anti-cancer drug, which shows quite high cytotoxic activity towards the normal cell line.

However, to date, there was no study that relates the cytotoxic effects of *E. Longifolia* towards nasopharyngeal carcinoma. Hence, this study aims to determine the cytotoxic activity of the *E. longifolia* extract against nasopharyngeal carcinoma.

MATERIALS AND METHOD

Preparations of *E. longifolia* root extracts

Root of *E. longifolia* was obtained from certified supplier. Soxhlet extraction procedure was performed by using ethanol and dichloromethane as solvents.

Maintenance and preparations of cancer cells for MTS assay

The cytotoxic activity of the extracts was evaluated at the Cell Culture Laboratory, Kulliyyah of Medicine at the IIUM Kuantan Campus. Nasopharyngeal carcinoma cell line (ORL-115) was obtained from Kulliyyah of Medicine, IIUM. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/high glucose supplemented with 10% Fetal Bovine Serum (FBS), 2 mM L-glutamine and 1% penicillin/streptomycin (Gibco, USA) under 5% CO_2 condition. Cells were seeded into six 96-well microtiter plates (in sixtuplicates) with 2 x 10^4 cells per well. PBS was added at the periphery wells to prevent moisture loss. The plates were incubated at 37°C for 24 h under a humidified atmosphere of 5% CO_2.

Treatment of cells with extracts

After 24 hours, the full cell medium (DMEM with 10% FBS) in test wells were changed to DMEM with 5% FBS (5% DMEM) containing 50, 100, 200, 400 and 800 µg/ml of the test extracts, while the control wells contained only 5% DMEM. The plates were then incubated for 24, 48 or 72 h. After each incubation period, 5% DMEM in control and test wells were replaced by 20 µL/well of MTS (Promega, Madison, WI, USA) and incubated at 37°C for an additional 3 h, followed by one minute of gentle shaking. The absorbance at 490 nm was read on a microplate reader.

STATISTICAL ANALYSIS

Data were presented as median ± IQR and analysed by using Kruskal-Wallis and Wilcoxon Signed Rank Test. Multiple comparisons was performed by using pairwise analysis (significance level $p < 0.05$) using software computer program (SPSS Statistics v.23.).

RESULTS

Cytotoxic activity of ethanol extract and DCM extract

In this study we investigated the cytotoxic activity of the *E. longifolia* extract against nasopharyngeal carcinoma. The cytotoxic effect of the root extracts against ORL-115 cell line were evaluated using MTS assay. IC_{50} is the inhibitory concentration of the extract that kills 50% of the nasopharyngeal carcinoma cell lines.

As observed in Figure 1, during 24-hours incubation period, ethanol extract showed more cytotoxicity against ORL-115 cell line in comparison to DCM. We observed that the IC_{50} of ethanol was 232.1 µg/ml which was lower than the IC_{50} of DCM. IC_{50} of DCM obtained was 678.87 µg/ml. However, when the highest concentration was used (800 µg/ml), DCM showed more cytotoxic effect compared to ethanol.

Based on the above figure, DCM extract showed less cytotoxicity when compared to ethanol extract. The IC_{50} obtained for DCM and ethanol were 136.71 µg/ml and 66.86 µg/ml respectively. Similar to the observation in Figure 1, at concentration of 800 µg/ml, DCM exhibited more cytotoxic effect compared to ethanol.

Based on the above figure, it can be noted that at 72-hours incubation period, as the concentration increases, the cytotoxic activity of both extracts become more comparable to each other. Starting from 200 µg/ml, the cytotoxic activity of DCM extract slightly exceeded the cytotoxic activity of the ethanol extract. The IC_{50} obtained for ethanol extract and DCM extract were 42.6 µg/ml and 73.72 µg/ml respectively.

To summarize, ORL-115 cell line was more sensitive to ethanol extract compared to DCM extract when low concentrations of the extracts were used. Only after the incubation period was prolonged to 72 hours did the cytotoxic activity of DCM extract exceed that of ethanol extract.

Statistical analysis of the cytotoxic activity of ethanol extract and DCM extract

Dose dependent cytotoxicity

There is a significant difference in cytotoxicity of ethanol between different concentrations. There is a significant difference in cytotoxicity of DCM between different concentrations.

Time dependent cytotoxicity

There is a significant difference in cytotoxicity of ethanol extract between different incubation time. There is a significant difference in cytotoxicity of DCM extract between different incubation time.

Dose dependent and time dependent cytotoxicity between two different extracts

DISCUSSION

Previous studies revealed that the cytotoxic activity of *E. longifolia* was attributed to the presence of numerous bioactive constituents such as eurycomalactone, longilactone, pasakbumin B, eurycomanone and β-carboline alkaloids. Different compounds possess different cytotoxic effects towards various type of cancer cell line [7].

In our study, both ethanol extract and DCM extract showed cytotoxic activity against ORL-115 cell lines. However, they displayed different potency in exerting the cytotoxicity towards the cell lines. The highest cytotoxic activity was observed in ethanol extract which exhibited lower IC_{50} value compared to DCM extract for each incubation period (Table 1). This different in potency might be due to different amount of bioactive constituents being yield by different type of extracts. DCM being less polar compared to ethanol might yield less amount of bioactive constituents.

In a study conducted by Nurhanan *et al.* [8], they investigated the cytototoxic effect of methanol, chloroform, n-butanol and aqueous extracts of the root of *E. longifolia* against various cancer cell lines which were ovarian cancer (Caov-3), prostate cancer (DU-145), epidermoid carcinoma (KB), rhabdosarcoma (RD), breast cancer (MCF-7) and bovine normal kidney (MDBK) cell lines. They found that the highest cytotoxic effect of the *E.longifolia* root extract was exerted by chloroform

extract and followed by methanol extract. This finding was parallel to the result of the chemical analysis whereby the chloroform extract was found to contain higher amount of 9-methoxycanthin-6-one, one of the bioactive constituents that was believed to be responsible for the cytotoxic effect of the extracts. Interestingly, in this study, chloroform was less polar compared to methanol. Thus, based on the result of our study and the previous study, we can say that appropriate solvents are needed in order to obtain extracts with high concentration of bioactive constituents that can exert cytotoxic activity against the cancer cell lines.

The cytotoxic activity of both extracts increased as the incubation time prolonged. This can be observed from figure 1, figure 2 and figure 3, whereby each concentration of each extract showed reduction in the percentage of survived cells as the incubation time prolonged. At 24 hours, ethanol extract killed more than 50% of the cell lines at lower concentration compared to DCM extract which needed higher concentration to kill 50% of the cell lines. DCM extract needed longer incubation time to kill 50% of the cells using lower concentration. To summarise, the cytotoxic activity of both extracts were time dependent and dose dependent. Ethanol extract needed shorter time and lower concentration to showed its cytotoxic effect compared to DCM extract.

Besides *E.longifolia*, a few studies had been conducted previously to investigate the cytotoxic effect of other natural products on nasopharyngeal carcinoma cell line. Qi *et al.* [9] used Cinnamic Acid (CINN) which is the main ingredient of the traditional Chinese medicine cinnamon to investigate its effect towards the proliferation and apoptosis of CNE2 human nasopharyngeal carcinoma cells. The cells were treated with different concentrations of CINN for 24-hour, 48-

Table 1: **IC_{50} of ethanol and dichloromethane (DCM) extracts for 24-hours, 48-hours and 72-hours incubation period.**

	24-hours	48-hours	72-hours
Ethanol	232.1 µg/ml	66.86 µg/ml	42.6 µg/ml
Dichloromethane	678.87 µg/ml	136.71 µg/ml	73.72 µg/ml

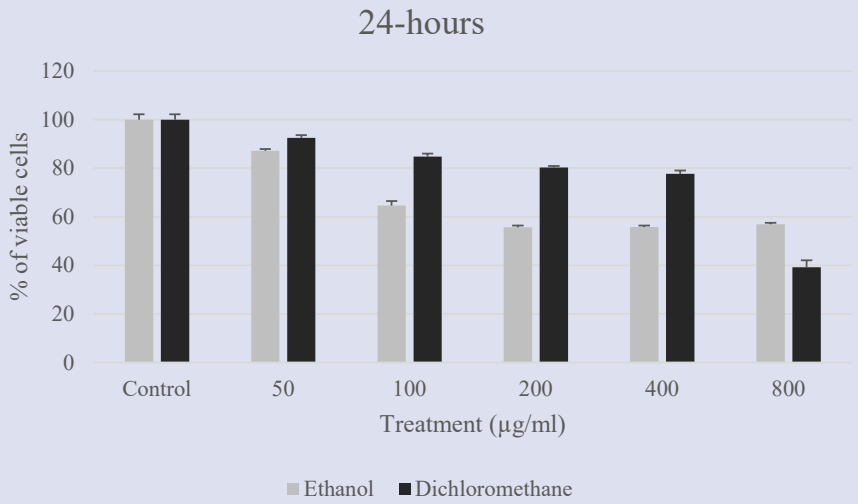

Figure 1: Cytotoxicity of ethanol and dichloromethane extracts of *E. longifolia* against nasopharyngeal carcinoma (ORL-115) cell lines at 24-hours incubation period. Values are shown as mean and error bars are standard deviation.

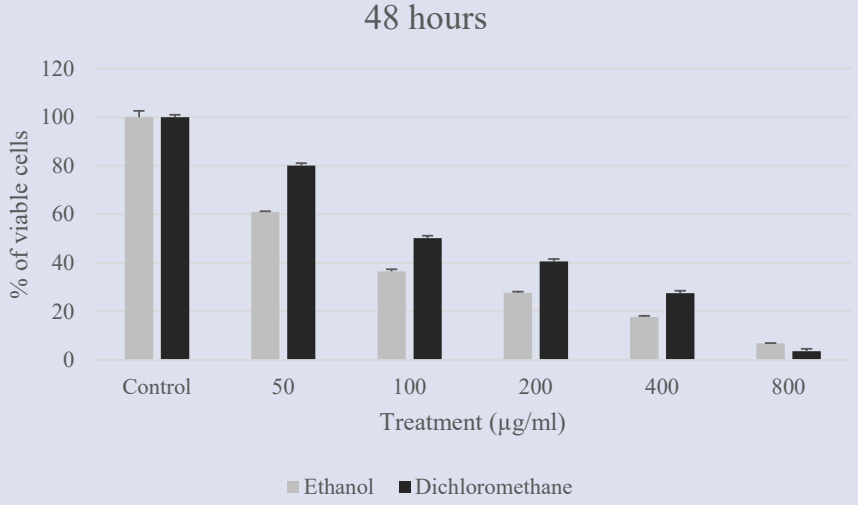

Figure 2: Cytotoxicity of ethanol and dichloromethane extracts of *E. longifolia* against nasopharyngeal carcinoma (ORL-115) cell lines at 48-hours incubation period. Values are shown as mean and error bars are standard deviation.

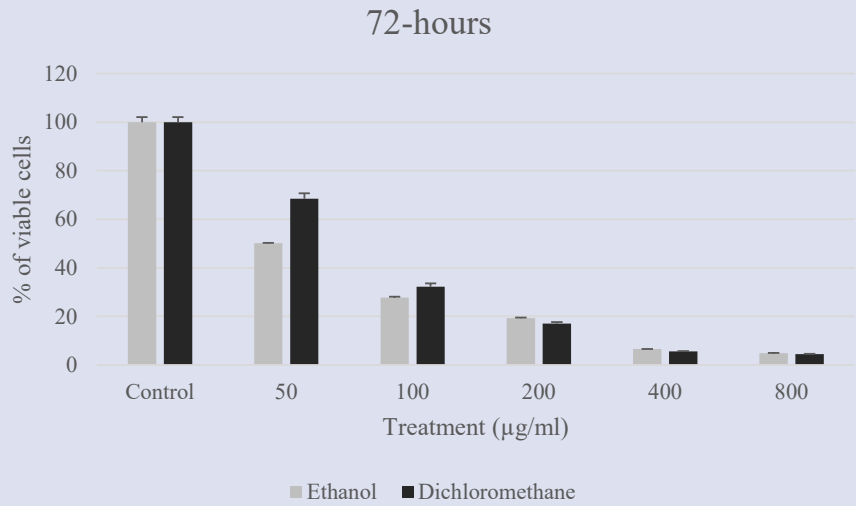

Figure 3: Cytotoxicity of ethanol and dichloromethane extracts of *E. longifolia* against nasopharyngeal carcinoma (ORL-115) cell lines at 72-hours incubation period. Values are shown as mean and error bars are standard deviation.

Table 2: Pairwise analysis: 800 µg/ml vs 400 µg/ml, 800 µg/ml vs 200 µg/ml, 400 µg/ml vs 200 µg/ml, 400 µg/ml vs 100 µg/ml and 200 µg/ml vs 100 µg/ml – p value > 0.05. Other pair comparisons p value < 0.05.

Concentration (µg/ml)	Median (IQR) Ethanol	F statistic (df)	p value
Control	2.004750 (0.4062)	65.449(5)	0.000
50	1.272600 (0.9500)		
100	0.747050 (0.8202)		
200	0.585350 (0.8041)		
400	0.374300 (1.0326)		
800	0.146200 (1.0877)		

Table 3: Pairwise Analysis: 400 µg/ml vs 200 µg/ml, 400 µg/ml vs 100 µg/ml, 200 µg/ml vs 100 µg/ml, 100 µg/ml vs 50 µg/ml and 50 µg/ml vs control – p value > 0.005. Other pair comparisons p value < 0.05.

Concentration (µg/ml)	Median (IQR) DCM	F statistics (df)	p value
Control	2.004750 (0.4062)	63.097 (5)	0.000
50	1.677500 (0.6638)		
100	1.074650 (1.1834)		
200	0.861450 (1.3720)		
400	0.588500 (1.4842)		
800	0.079850 (0.6837)		

Table 4: Pairwise Analysis: All pair comparisons p values < 0.05.

Time (hour)	Median (IQR) Ethanol	F statistic (df)	p value
24	1.239500 (0.6314)	34.556 (2)	0.000
48	0.669150 (0.9034)		
72	0.391350 (0.7316)		

Table 5: Pairwise Analysis: All pair comparisons p values < 0.05.

Time (hour)	Median (IQR) DCM	F statistic (df)	p value
24	1.723450 (0.2803)	24.051(2)	0.000
48	0.925950 (1.0907)		
72	0.392600 (1.0341)		

Table 6: The cytotoxic activity of ethanol extract at each concentration is significantly different from DCM extract.

Concentration (µg/ml)	Median (IQR)		Z statistic	p value
	Ethanol	DCM		
Control	2.0048 (0.4061)	2.0048(0.4061)	0.000	1.000
50	1.2726 (0.9500)	1.6775 (0.6638)	-3.727	0.000
100	0.7470 (0.8203)	1.0747 (1.1834)	-3.680	0.000
200	0.5854 (0.8041)	0.8615 (1.3720)	-2.809	0.005
400	0.3743 (1.0326)	0.5885 (1.4842)	-2.809	0.005
800	0.1462 (1.0877)	0.0799 (0.6836)	-3.680	0.000

Wilcoxon Signed Ranks Test

Table 7: The cytotoxic activity of ethanol extract at each incubation time is significantly different from DCM extract. However, there was no significant difference between the cytotoxic activity of both extracts at 72-hours.

Time (hour)	Median (IQR)		Z statistic	p value
	Ethanol	DCM		
24	1.2340 (0.6314)	1.7235 (0.2803)	-3.075	0.002
48	0.6692 (0.9033)	0.9260 (1.0906)	-4.351	0.000
72	0.3914 (0.7316)	0.3926 (1.0341)	-0.833	0.405

Wilcoxon Signed Ranks Test

hour and 72-hour. Similar to our results, they found out that the growth of CNE2 cells were significantly inhibited with increasing concentration of CINN and treatment time. Another recent study done by Liu *et al.* [10] also found that brevilin A that was isolated from *Centipeda minima* exerted cytotoxic effect on nasopharyngeal carcinoma cell lines in a dose and time dependent manner. Unfortunately, there was no study done previously to investigate the time dependency of *E. longifolia* extract towards nasopharyngeal carcinoma. Hence, our results serve as a new information for the study of natural product in the treatment of nasopharyngeal carcinoma.

CONCLUSION

Our results demonstrate that *E. longifolia* root extracts exerted cytotoxic activity against the nasopharyngeal carcinoma (ORL-115) cell line. Ethanol extract exhibited lower IC_{50} value compared to DCM extract. The cytotoxic activity of both extracts were dose dependent and time dependent. Hence, it is suggested that ethanol extract has better cytotoxic potential since it is rapidly acting and requires lower concentration to kills more cells compared to DCM. The use of lower concentration is better to avoid any unwanted toxic effect towards normal cells. The potential of *E. longifolia* as cytotoxic drug towards nasopharyngeal carcinoma can be further studied through the identification of the responsible bioactive component and followed by *in vivo* study.

ACKNWOLEDGEMENT

We would like to thank the University Malaysia Pahang and International Islamic University Malaysia for funding this work through the sustainable research collaboration grant number: SRCG20-025-0025.

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Cite this article: Kajahmohideen NH, Razi SN, Faisal GG, Ashour AE, Kusumawardani A, Makky EA, et al. Cytotoxic Activity of Eurycoma Longifolia Jack Root Extract Against Nasopharyngeal Carcinoma Cell Line. Pharmacogn J. 2021; 13(4): 1014-1018.