
**Faculty of Medicine and Health Sciences**

# *In-vitro* Study of Cytotoxicity and Apoptosis Effects of *Clinacanthus nutans* (Burm.f.) Lindau Extracts on Colorectal Cancer Cell Lines, HT-29 and HCT-116

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# *In-vitro* Study of Cytotoxicity and Apoptosis Effects of *Clinacanthus nutans* (Burm.f.) Lindau Extracts on Colorectal Cancer Cell Lines, HT-29 and HCT-116

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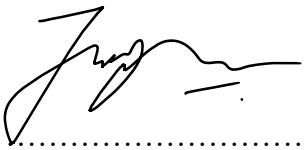
## DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.



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## ABSTRACT

Cancer is one of the leading causes of mortality in Malaysia, with colorectal cancer as the most common cancer in males and the second most common cancer in females. Colorectal cancer causes high mortality as it is hardly detected during the early stage yet has a 15-year window of intervention. Hence, the discovery of the chemoprevention method is important to delay carcinogenesis and prevent the recurrence of cancer. *Clinacanthus nutans* (Burm.f.) Lindau (*C. nutans*), from *Acanthaceae* family, is known as Sabah Snake Grass or '*Belalai Gajah*' in Malaysia. It is popular in Southeast Asia for its medicinal properties such as anticancer, anti-inflammation and antiviral properties. Therefore, this study aimed to determine the total phenolic content (TPC), total flavonoid content (TFC), antioxidant activity, and anticancer properties of *C. nutans* leaves extracts on human colorectal cancer cell lines, HCT-116 and HT-29 in dose- and time-dependent manners. The ground *C. nutans* leaves were extracted with methanol, chloroform, and acetone for 30 minutes and 24 hours, respectively. The TPC and TFC were determined spectrophotometrically using the Folin-Ciocalteu and aluminium chloride colourimetric methods. 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging assay was used to determine the antioxidant activity. The antiproliferation and apoptotic capabilities were studied using MTT and apoptosis (Annexin V-FITC and PI staining method) assays, respectively. *C. nutans* methanol extracts and 24 hours of extraction time resulted in higher extraction yield (7.65g ± 0.13) than its counterparts. Acetone extracted *C. nutans* at 30 minutes and 24 hours resulted in the highest TPC values of 3.06 mg GAE/g and 3.18 mg GAE/g, respectively. On the other hand, methanol extracted *C. nutans* at 30 minutes and 24 hours exhibited the highest TFC (0.49 mg QE/g and 0.50 mg QE/g, respectively) and strongest antioxidant activity ($IC_{50}$ values at 24.25 $\mu$g/mL and 19.67 $\mu$g/mL; AAEAC at 22.14% and 27.31%, respectively) than

chloroform and acetone extracts. The methanol extracts were selected for *in vitro* study. HCT-116 cells revealed higher antiproliferative effects than HT-29 cells using methanol extracted *C. nutans* at 30 minutes when treated for 24 hours and 72 hours. On the contrary, HCT-116 cells exhibited lower antiproliferative effects when treated for 48 hours. Methanol extracted *C. nutans* at 24 hours exhibited stronger antiproliferation activity when treated on HT-29 cells with less cytotoxicity to normal cells for 24, 48, and 72 hours. In addition, a low dose of 250 $\mu$g/mL methanol extracted *C. nutans* with 24 hours extraction time induced early and late apoptosis in HCT-116 and HT-29 cells after treatment for 72 hours. The methanol extract increased the late apoptosis of HCT-116 cells from 18.88% to 35.66% when treatment from 24 hours to 72 hours, respectively. The late apoptotic rate of HT-29 cells was increased from 14.28% to 20.67% when treatment was prolonged from 24 hours to 72 hours, respectively. In conclusion, methanol extracted *C. nutans* that underwent 24 hours extraction contained high TFC, exhibited high antioxidant activity, and able to inhibit the early and late stages of colorectal cancer cell growth by triggering apoptosis in dose- and time-dependent manners. The findings suggested that *C. nutans* possessed anticancer properties against colorectal cancer cell lines, HCT-116 and HT-29 through the apoptosis pathway. The findings provided new perspective for the future study to prove *C. nutans* as an alternative treatment against early and late stages colorectal cancer.

**Keywords:** *Clinacanthus nutans*, colorectal cancer, antioxidant, anticancer, apoptosis

***Kajian In-vitro tentang Kesan Sitotosisiti dan Apoptosis Clinacanthus nutans (Burm.f.) Lindau pada Sel Kanser Kolorektal, HT-29 dan HCT-116***

## *ABSTRAK*

*Kanser adalah salah satu punca utama kematian di Malaysia dengan kanser kolorektal sebagai kanser paling biasa di kalangan lelaki dan kanser kedua paling biasa di kalangan wanita. Kanser kolorektal menyebabkan kematian yang tinggi kerana ia jarang dikesan pada peringkat awal tetapi mempunyai tempoh intervensi selama 15 tahun. Oleh itu, penemuan kaedah kemopencegahan adalah penting untuk melambatkan karsinogenesis dan mencegah berulangnya kanser. Clinacanthus nutans (Burm.f.) Lindau (C. nutans ), daripada keluarga Acanthaceae, dikenali sebagai 'Sabah Snake Grass' atau Belalai Gajah di Malaysia. Ia popular di Asia Tenggara dengan khasiat perubatannya seperti antikanser, anti-radang dan antivirus. Oleh itu, kajian ini bertujuan untuk menentukan jumlah kandungan fenolik (TPC), jumlah kandungan flavonoid (TFC), aktiviti antioksidan, dan sifat antikanser ekstrak daun C. nutans pada sel kanser kolorektal manusia, HCT-116 dan HT-29 dengan cara yang bergantung kepada dos dan masa. Daun C. nutans yang telah dikisar, diekstrak dengan metanol, kloroform, dan aseton selama 30 minit dan 24 jam. TPC dan TFC ditentukan secara spektrofotometri dengan menggunakan kaedah Folin-Ciocalteu dan kolorimetrik aluminium klorida. Ujian penghapusan radikal 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) digunakan untuk menentukan aktiviti antioksidan. Kemampuan antiproliferasi dan apoptosis telah dikaji melalui ujian MTT dan apoptosis (kaedah pewarnaan Annexin V-FITC dan PI). Ekstrak metanol C. nutans dan 24 jam masa pengekstrakan menghasilkan hasil pengekstrakan yang lebih tinggi (7.65g ± 0.13). Ekstrak aseton C. nutans pada 30 minit dan 24 jam menghasilkan nilai TPC tertinggi iaitu 3.06 mg GAE/g dan 3.18 mg GAE/g, masing-masing. Sebaliknya, ekstrak metanol C. nutans pada 30 minit dan 24 jam*

*menunjukkan TFC tertinggi (0.49 mg QE/g dan 0.50 mg QE/g) dan aktiviti antioksidan tertinggi ($IC_{50}$ pada 24.25 μg/mL dan 19.67 μg/mL; AAEAC pada 22.14% dan 27.31%, masing-masing) daripada ekstrak kloroform dan aseton. Ekstrak metanol kemudiannya dipilih untuk meneruskan kajian in vitro. HCT-116 mendedahkan kesan antiproliferasi yang lebih tinggi berbanding sel HT-29 menggunakan ekstrak metanol C. nutans dalam 30 minit apabila dirawat selama 24 jam dan 72 jam. Sebaliknya, sel HCT-116 mempamerkan kesan antiproliferasi yang lebih rendah apabila dirawat pada 48 jam. Selain itu, ekstrak metanol C. nutans dalam 24 jam menunjukkan aktiviti antiproliferasi yang lebih kuat apabila dirawat pada sel HT-29 dengan kurang toksik kepada sel normal selama 24, 48, dan 72 jam. Di samping itu, dos rendah 250 μg/mL ekstrak metanol C. nutans dalam 24 jam menyebabkan apoptosis awal dan lewat dalam sel HCT-116 dan HT-29 selepas rawatan selama 72 jam. Ekstrak metanol meningkatkan apoptosis lewat sel HCT-116 daripada 18.88% kepada 35.66% apabila rawatan daripada 24 jam kepada 72 jam, masing-masing. Selain itu, apoptosis lewat sel HT-29 meningkat daripada 14.28% kepada 20.67% apabila rawatan daripada 24 jam kepada 72 jam, masing-masing. Kesimpulannya, ekstrak metanol C. nutans pada 24 jam mengandungi TFC yang tinggi dan mempamerkan aktiviti antioksidan yang tinggi di samping dapat menghalang peringkat awal dan akhir pertumbuhan sel kanser kolorektal dengan mencetuskan apoptosis dalam cara yang bergantung kepada dos dan masa. Penemuan mencadangkan bahawa C. nutans mempunyai sifat antikanser terhadap garisan sel kanser kolorektal, HCT-116 dan HT-29 melalui laluan apoptosis. Penemuan ini memberikan perspektif baru untuk kajian masa depan untuk membuktikan C. nutans sebagai rawatan alternatif terhadap kanser kolorektal peringkat awal dan akhir.*

***Kata kunci:*** *Clinacanthus nutans, kanser kolorektal, antioksidan, antikanser, apoptosis*

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| | |
|---|---|
| ABTS | 2, 2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid |
| *C. nutans* | *Clinacanthus nutans* |
| CRC | Colorectal cancer |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| DPPH | 2,2-Diphenyl-1-picrylhydrazyl |
| FACS | Fluorescence-activated cell sorting |
| FITC | Fluorescein isothiocyanate |
| FRAP | Fluorescence recovery after photobleaching |
| IFN-γ | Interferon gamma |
| IL | Interleukin |
| HCT-116 | Human epithelial colorectal carcinoma cell line |
| HT-29 | Human epithelial colorectal adenocarcinoma cell line |
| LPS | Lipopolysaccharide |
| MDA | Malondialdehyde |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| NO | Nitrite oxide |
| PBS | Phosphate-buffered saline |
| PI | Propidium iodide |
| PS | Phospholipid phosphatidylserine |
| RNS | Reactive nitrogen species |
| ROS | Reactive oxygen species |
| SRB | Sulforhodamine B |

| | |
|---|---|
| TFC | Total flavonoid content |
| TLR-4 | Toll-like receptor 4 |
| TNF-$\alpha$ | Tumour necrosis factor alpha |
| TPC | Total phenolic content |

# CHAPTER 1

# INTRODUCTION

## 1.1 Background of Study

Cancer is one of the leading causes of non-communicable disease mortality with approximately 10 million mortalities worldwide in 2020 (WHO, 2022). World Health Organisation (WHO) reported that colorectal cancer was the third most common cancer and second most common cause of global mortality in 2020 (WHO, 2022). In Malaysia, colorectal cancer was the most common cancer in males and the second most common cancer in females, as stated in the Malaysia National Cancer Registry Report 2012-2016 (Azizah et al., 2019). Due to the hidden nature of its site, it is often left undetected until it is too far along with its progression and formation of hyperplastic and gradually dysplastic cells. These cells eventually become aberrant crypt foci (ACF) and polyps. Undetected polyps lead to a later stage of malignant carcinomas, also known as colorectal cancer (Pandurangan et al., 2018). At present, the routine treatment and management regime include surgical removal of tumour, chemotherapy, radiotherapy, or a combination of these three procedures. These procedures come with massive burdens in terms of human capital, cost of treatment and hospitalisation, and oftentimes, lives (Veettil et al., 2017; Costea et al., 2018). Therefore, it is pertinent to searching for alternative, less invasive treatments with low or no side effect intervention as a chemoprevention method against colorectal cancer.

## 1.2 Plants as Chemopreventive Agents

Chemoprevention is known to slow down and inhibit carcinogenesis or prevent the reappearance of cancer by using natural products or synthetic molecules (Siddiqui et al., 2015; Melo et al., 2018). Chemopreventive agents inhibit carcinogens from affecting DNA, aid in repairing DNA, delay the cell cycle process, and inhibit metastasis by affecting the related events (Melo et al., 2018).

There are three types of chemopreventive agents such as cancer formation inhibitors, suppressing agents, and blocking agents (**Figure 1.1**). Chemopreventive agents that act as inhibitors prevent the formation of cancer while suppressing agents exhibit antiproliferation effects by triggering apoptosis, necrosis, and autophagy to suppress the cancer progression. Blocking agents interfere with the carcinogenesis pathways and capture certain reactive oxygen species (ROS) by inducing the detoxification process (George et al., 2021). Chemopreventive compounds scavenge free radicals or activate enzymes that require scavenge free radicals to inhibit DNA alteration by a carcinogen (Melo et al., 2018). ROS and reactive nitrogen species (RNS) are intracellular free radicals usually produced in cell metabolic activities as by-products (Yong et al., 2013). Oxidative stress is occurred when ROS production is increased due to environmental factors and intracellular ROS (Aggarwal et al., 2019). Oxidative stress tends to cause cancer, diabetes mellitus, chronic inflammation, and atherosclerosis (Valko et al., 2007).

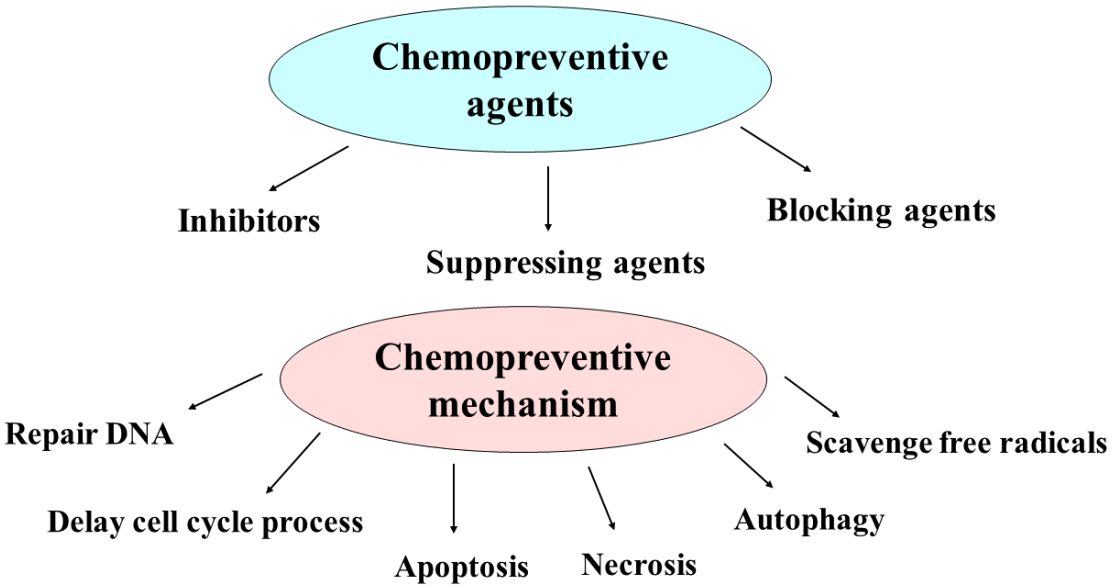


**Figure 1.1:** Types of chemopreventive agents and examples of chemopreventive mechanism.

Medicinal plants have been reported to elicit numerous efficacious benefits to our health (Zulkipli et al., 2017). When these are consumed or applied topically, a plethora of phytochemicals or naturally produced secondary metabolites exhibit medicinal properties, including anticancer, anti-inflammatory, and antimicrobial activities (Zulkipli et al., 2017). Some phytochemicals are natural antioxidants that work as free radical acceptors and hydrogen donors to suppress the oxidation reaction (Shahidi & Zhong, 2015). The antioxidant activity of phytochemicals is beneficial to our health as it can eliminate the excess ROS or RNS and stabilise the oxidant level in our body. Furthermore, bioactive compounds in plants have also been proven to trigger apoptosis, suppress cancer growth, and arrest the cell cycle (Zlotogorski et al., 2013; Schaffer et al., 2015; Sheikh et al., 2017a; Sheikh et al., 2017b; Tungmunnithum et al., 2018). Paclitaxel, also known as Taxol, is a common chemo drug that is derived from the bark of *Taxus brevifolia* Nutt. (Ashraf, 2020). Another example of anticancer compound that is derived from medicinal plant is vinblastine

from *Catharanthus roseus* G. Don. (Ashraf, 2020). Vinblastine in combination with vincristine and other chemo drugs is commonly used in a wide range of cancers especially lung cancer, breast cancer, and leukaemia (Ashraf, 2020). Therefore, the search for natural promising chemopreventive agents against cancer formation and metastasis provides an alternative to these expansive colorectal cancer treatments is strongly justified.

Phenolic compounds play important roles in inhibiting carcinogenesis stages; they are popular in chemoprevention (George et al., 2021). Phenolic compounds are common secondary metabolites in plants such as vegetables, fruits, and tea. The structure of phenolic compounds contains non-polar and polar components. The non-polar component is an aromatic ring with an attachment of polar part, which is single or multiple hydroxyl groups (Queimada et al., 2009). Phenolic compounds have strong antioxidant properties, and they are divided into flavonoid, and non-flavonoid based on the chemical structure (Costea et al., 2018). Examples of flavonoids are flavanones, isoflavonoids, flavonols, flavones, anthocyanidins, and flavonols (Manach & Donovan, 2004; Durazzo et al., 2019; Mark et al., 2019), whereas non-flavonoids are lignans, tannins, stilbenes, and phenol carboxylic acids (Caleja et al., 2017; Domínguez-Avila et al., 2017; Costea et al., 2018). Quercetin, a type of flavonol contained in apples and onion, has been reported to possess anticancer effects against colorectal cancer by downregulating altered genes and the synergistic manner in combination with chemo drugs (Costea et al., 2018).

### 1.2.1 Clinacanthus nutans

*Clinacanthus nutans* (*C. nutans*), from the Acanthaceae family, is also known as Sabah Snake Grass in English and '*Belalai Gajah*' in Bahasa Malaysia. *C. nutans* is a perennial herb that can grow between 1 to 3 m tall with pubescent branches and smooth,

striate, and cylindrical stems. It can be widely found in Southeast Asia and China (Yahaya et al., 2015; Alam et al., 2016; Zulkipli et al., 2017). The leaves are long and narrowly V-shaped with alternate opposite leave arrangements (Alam et al., 2016; Zulkipli et al., 2017). It is a reputable medicinal plant in Southeast Asia and is widely used in Thailand and Malaysia in treating inflammation, some cancers, and *Herpes simplex* virus infections (Zulkipli et al., 2017). It is commonly brewed and consumed as herbal tea in Malaysia, particularly treat diabetes (Alam et al., 2016). In addition, fresh leaves are also blended with apple, green tea, or sugarcane prior to consumption (Yahaya et al., 2015). It is also used topically as anti-snake venom in Thailand as it has anti-cell lysis properties (Alam et al., 2016). The ethnobotany uses of *C. nutans* from different geographical regions were summarised in **Table 1.1**.

**Figure 1.2:** *Clinacanthus nutans*

**Table 1.1:** Ethnobotany uses of *C. nutans* from different geographical regions

| Preparation method | Effect | Regions | Reference |
|---|---|---|---|
| Boiled fresh leaves with water. | Treatment for diabetes | Malaysia | Alam et al., 2016<br>P'ng et al., 2013<br>Yahaya et al., 2015 |
| Served as juice by blending the leaves. | Anti-cancer | | |
| Alcohol extraction of leaves are applied on skin. | Treatment for varicella-zoster virus (VZV), herpes simplex virus (HSV) lesions, skin rashes, and insect and snake bite. | Thailand | Sookmai et al., 2011<br>Alam et al., 2016 |
| Consumed as raw or blended with sugarcane, juice, or green tea | Anti-snake venom | | |
| | Treatment for scorpion bites and nettle rash | | |
| A handful of leaves is cooked in five glasses of water. | Treatment for dysentery, diabetes, dysuria, and fever | Indonesia | Alam et al., 2016 |
| About 7 to 21 leaves is boiled in two glasses of water. | | | |
| Decoction of leaves. | | | |

## 1.3 Problem Statement

Colorectal cancer is one of the leading causes of death in worldwide. The development of polyp to malignant colorectal cancer takes 10 to 15 years (Hossain et al., 2022). From this long-term malignancy development, it is believed that there is an opportunity for immediate treatment before progressing into late stage of cancer. Previous expansive studies on *C. nutans* revealed its pharmacological activities, such as antioxidant, antiviral, anti-inflammation and anticancer (Zulkipli et al., 2017). For example, previous studies showed that *C. nutans* extract inhibited the cell growth of colorectal cancer cell line, HCT-116 and induced apoptosis in cervical cancer cell, HeLa (Esmailli et al., 2016; Haron et al., 2019). Therefore, it is important to study the purported medicinal potential of *C. nutans* in treating colorectal cancer by determining the anticancer effects on early and late stage of colorectal cancer cells.

## 1.4 Objectives of Study

This study has achieved the following specific objectives:

i. To determine the total phenolic content (TPC), total flavonoid content (TFC) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of different *C. nutans* solvent extracts.

ii. To assess the antiproliferation activity of *C. nutans* extracts against HCT-116 and HT-29 cell lines using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in time- and dose-dependent manners.