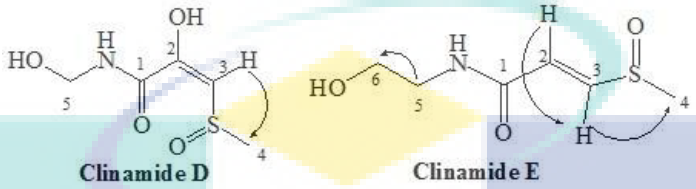
BUKU PROFIL PENYELIDIKAN SKIM GERAN PENYELIDIKAN FUNDAMENTAL (FRGS) FASA 1/2014



ISOLATION AND CHARACTERIZATION OF APOPTOSIS-INDUCING COMPOUNDS FROM CLINACANTHUS NUTANS

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ABSTRACT (120 words)

Clinacanthus nutans have been used in traditional herbal medicine for cancer prevention but specific bioactive compounds responsible for the observed activities have not been explored. Different solvents such as methanol, chloroform, ethyl acetate and hexane were used for the extraction. The extracts were subjected to in vitro antioxidant and anticancer activities. Phytochemical screening was done by determining total phenolic content (TPC) and total flavonoid content (TFC). The methanol extract of *C. nutans* showed significant antioxidant activity for 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity (IC50 127.09± 0.042 µg/mL), total reduction capability (IC50 469.13 ± 0.05 µg/mL and anticancer activity on HT29 (IC50 36.19 ± 1.06 mg/mL), HepG2 (IC50 48.37 ± 0.026 mg/mL) and MCF-7 (IC50 54.16 ± 0.99 mg/mL) after 72 h of treatment. Total phenolic content (TPC) was found to be highest in chloroform, which was 119.29 mg of gallic acid equivalent (GAE) a nd total flavonoid content (TFC), methanol was performed the highest value, which is 937.67 mg of butylated hydroxytoluene (BHT). Purification of MeOH extracts afforded two new sulphur-containing compounds, named clinamide D and E (1-2). Compound (1) proved to be more active with IC_{50} value for DPPH radical scavenging of 118.27± 0.01µg/mL and reduction of Fe^{3+}–TPTZ complex of 386.24 ± 0.02, higher than that standard ascorbic acid. Sulphur-containing compounds isolated from. *C. nutans* is a potential source as a natural antioxidant.

1. INTRODUCTION

1.1 Research Background

Natural products have played a very important role as medicine and prevention of diseases. Natural product is a chemical compoundsproduced by the organism such as a plant, a fungus, a bacterial species or even marine creatures [1] found in the nature that are produced by the pathways of primary and secondary metabolism. [2]. Synthetic chemical compoundswere designed based on isolated compounds from the nature.Natural products have been the major sources of chemical diversity in pharmaceutical discovery over the past centuries (Baker et al., 2007).In addition, natural product also has vital role in pharmacological area and commercial industries which provide a lot of health care and medicinal product like antibacterial, anti-hepatotoxic, nutraceuticals, flavour and food additives. [3]

Ancient civilisation conducted various researches usingplants and animal parts in order to knowtheir biological effects.. Crude extractss were found to have healing power in reducing diseases. The biological effect of crude extractsmay lead the discovery of new bioactive compounds and contribute to a drugs development (Patwardhan et al., 2004)However, based on previous reports, a very little effort has been made to establish the scientific basis of traditional medicine and develop the avaibality of the plants into useful and valuable pharmaceutical products. (Yuan et al., 2016).

According to the World Health Organization (WHO), around 80% of world population in developing countries are used plant as a souces for the treatment of various diseases and ailments such as cancer treatment, anti-inflammatory, malaria, and other chronic disease such as cardiovascular, heart diseases, hypertension (Yahaya et al., 2015). The investigation of bioactive natural products were mainly concerned with discovering of bioactive constituents. A research on isolation and characterization of pharmacologically active compounds from plants continue until today. .

Medicine plants secondary metabolites that derived biosynthetically from plant primary metabolites (e.g., carbohydrates, amino acids, and lipids) (Gulfraz et al., 2004). Plants produce primary metabolites as agrowth function (Kabera et al. 2014).andsecondary metabolites to protect the plant [4]Secondary metabolites can be categorised into three main groups according to their biosynthetic origin which are terpenes, nitrogen-containing alkaloids and phenolic compounds. They act as defences chemicals and does not cause harm effect to the plants (Kabera et al., 2014). [5] Terpenes or isoprenoids are the largest family of natural products, and possess at least one 5- carbon (C5) isoprenes units. Steroids, carotenoids and gallicc acid are some of the members. [4]. The secondary largest secondary metabolites group is nitrogen-containing alkaloids which structurally diverse group of compounds, and commonly distinguished from amines. Other than that, most of them have a heterocyclic nitrogenous rings or ring system. (Kabera et al., 2014 and Fong, 2015)The third largest group is the phenolic compounds which constituents synthesized by fruits, vegetables, teas, cocoa, and other plants. They are characterised by having at least one aromatic ring bearing one or more hydroxyl groups, which can undergo esterification, methylation, and other reactions. According to Kabera et al. (2014), phenolic compound can divided into four main groups phenolic with one aromatic ring, with two aromatic ring, quinones and polymers. (e.g., phenolic acids, flavonoids, stilbenes, coumarins, quinones, lignans, curcuminoids and tannins)

All these compounds have their own therapeutic effects and possess significant pharmacological activities. This information on medicinal plants helps researcher in providing scientific basis for their properties. For example, terpenes tend to have antimicrobial and antiviral properties. [6] and some interesting compounds are used in the industry sector as flavours, fragrance or spices. Other than that, phenolic compounds also possess certain health benefits such as antioxidant, anti-inflammatory and anti-cariogenic[7].

Cancer still remains in contributing to be one of the major health problems in Malaysia populations. Tumorigenesis is closely related with the level of intracellular free radicals which reactive oxygen/ nitrogen (RONS) which can trigger the growth and progression of cancer cell [8]. RONS reacted with other substance in the body will cause damage and dangerous to tissue

injury also promotes the formation of cancer cell [9]. Chemical based antioxidants are used to control free radical activity and development of natural antioxidants would be benefit to humankind. Hence, natural antioxidants compounds that purified from plants products could save cells from oxidative stress and prevent the growth of cancer cell (Kuppusamy et al., 2015). Polyphenols are the largest antioxidant constituents isolated from many medicinal plants which major mechanism of phenolic compounds is caused by their electron or hydrogen-donating ability (Ahn et al., 2012). .

1.2 Problem Statement

Cancer is the most distressing and life-threatening disease that enforces severe death worldwide (Krishnakumar et al., 2009). Moreover, Cancer remains as one of the major health threats to Malaysian people with yearly mortality rate of cancer patients that has consistently reached 10-11% since 2006 (Yong et al., 2013). Current treatments including surgery, radiotherapy, and chemotherapy are mostly ineffective against advanced stages of cancer, and they are also often associated with severe side effects. (Krishnakumar et al., 2009) [10]

Current drugs used for the cancer treatment are not selective of cancerous cells and their therapeutic efficiency is limited due to the damage they can cause to healthy cells and tissues. To avoid these side effects in cancer therapy, there is an urgent need to develop therapeutic modalities with no or minimal side effects to normal organs. The used of plants for therapy is not new; indeed plants have been considered a valuable source of bioactive compounds for treatment of many conditions, including cancer, in almost all cultures and communities for thousands of years (Ochwang'i et al., 2014).

Nowadays, there is a lot of research that has been reported the important of medicinal plants for healing process compare to the existence of medicinal synthesize chemicals. *C. nutans* is one of herbs that had been used in Thailand as a traditional medicine for the treatment of serious diseases (Aslam et al., 2014). The plant material and crude extract of *C. nutans* become

more popular nowadays as commercial products in Malaysia and Thailand as an antioxidant supplement. *C. nutans* has attracted Malaysian public interest due to its high medicinal values for the treatment of cancer. Investigations on*C. nutans* extract have been extensively studied a few years ago. There are various studies related to the phytochemicals, cytotoxicity and antioxidant activities of crude extracts from *C. nutans* . Unfortunately, there are no reports investigating thoroughly for the effect of specific compound on anticancer and antioxidant activity. Thus, in this study, we are attempting to isolate and elucidate the structure of anticancer and antioxidant compounds by using bioassay-guided fractions. The characterization of the potential compounds will be useful for the development of new drugs to treat cancer.

1.3 Objective of Research

This research is directed towards the study of *C. nutan* extracts including the isolation, identification, and characterisation of the compounds as well as the biological activities, the specific objectives of this research are:

1. To evaluate antioxidant properties and anticancer activity of extract and isolated compounds of *C.nutans*
2. To isolate compounds from *C. nutans* extract
3. To elucidate the structure of compounds by spectroscopic techniques

1.4 Scope of Study

In this study, *C. nutans* will be extracted methanol. The extract will be evaluated on the antioxidant properties and cytotoxicity effects. Antioxidant were evaluated using DPPH and FRAP assays while cytotoxicity was tested against human hepatoma (HepG2) cancer cell line using MTT assay. Phytochemical screening was done to determine the total phenolic content

(TPC) and total flavonoid content (TFC). The extract will undergo a purification and isolation process by chromatographic techniques.Characterisation of isolated compounds was carried out by using spectroscopic methods such as IR, NMR, (^{1}H. ^{13}C, DEPTQ, COSY, HMBC, HMQC) and MS.

2. RESEARCH METHODOLOGY

2.1 Extraction and Isolation

Powdered, dried *C. nutans* (5.0 kg) were defatted by percolation with hexane and filtration. The plant material was dried, extracted through three cycles of sonication (30 min each) with MeOH, filtered to obtain a dark green extract, and was concentrated under low pressure. MeOH extract (18.311 g) was chromatographed using a column with silica gel slurry packed in $CHCl_3$. Elution was initiated with $CHCl_3$, with progressively increasing concentrations of MeOH in $CHCl_3$, until it reached 100% MeOH. This protocol yielded twenty-one sub-fractions (F1–F21). F8 (2.31 g) (664.5 mg) was chromatographed with $CHCl_3$-MeOH (60:40) as eluting solvent to afford twenty-one subfractions (FA1-FA21). Sub fraction FA5 (128.5 mg), which were re-chromatographed with $CHCl_3$- MeOH (80:20) yielded compound (1**)** (1.71 mg). Sub fraction FA9 was chromatographed with $CHCl_3$-MeOH (85:15) to afford sub fraction FB28 and compound (2**)** (24.7 mg).

2.2 DPPH free radical scavenging assay

The potential antioxidant activities of *C.nutans* extracts and isolated compounds

were determined using the method described by Foon, Ai, Kuppusamy, Yusoff and Govindan [11] with some modifications. A stock solution of plant extracts and isolated compounds were prepared in different aliquots (31.25, 62.5, 125, 250, 500, and 1000 µg/mL) and each of them (100 µl) was mixed with 100 µl of DPPH methanolic solution (0.16 mM) in a 96-well plate. The mixtures were swirled gently for 1 min and kept in a dark room for 30 minutes. The absorbance of the samples was read at 540 nm in triplicate using the Tecan Infinite M200 PRO microplate reader at 540 nm. BHT was used as a reference compound in this assay. The ability of scavenging the DPPH radical was calculated with the following equation: % inhibition= $[(A_{control} - A_{sample}) / A_{control}] \times 100$.

2.3 Ferric Reducing Antioxidant Potential Assay (FRAP Assay)

FRAP assay was measured according to the method described by Benzie and Strain [12] with some modifications. The FRAP reagent was freshly prepared with 30 mmol/L acetate buffer (pH3.6), 10 mmol/L 2,4,6-Tri(2-pyridyl)-1,3,5-triazine, 40 mmol/L HCl and 20 mmol/L $FeCl_3$, 20 mmol/L $FeCl_3.6H_2O$ to the ratio of 10:1:1. This method is based on reduction of a ferric 2,4,6- Tri(2-pyridyl)-1,3,5-triazine complex (Fe^{3+}-TPTZ) to its ferrous (Fe^{3+}-TPTZ), intensive blue coloured form in the presence of antioxidant. The reagent solution was warmed to 37^0C before use . 20 µL of *C.nutans* extracts and isolated compound were mixed with 200 µL of reagent into 96- well plates. The mixture was left for 10 min and the absorbance was read at 593 nm using a Tecan Infinite M200 PRO microplate reader. Ascorbic acid was used as controlled item. The ability of the samples to scavenge free radicals was calculated and was reported as % scavenging rate = $(1 - A_{sample}/A_{control}) \times 100$.

2.4 Anticancer activity

Cells viability was determined by trypsin blue before plating. 80-90% confluent culture were harvested with 0.05% (v/v) trypsin 0.02% (v/v) EDTA and plated onto 96 well plates at initial density of approximately $5x10^4$ cells/mL and were left to be attached overnight. After overnight incubation, the medium was removed and replaced with fresh media (198 µl/well) with 2 µL of different aliquots plant extracts. Crude extract samples were prepared at 10 mg/mL. The testing solution was done by 2 times serial dilution for 10 points ranged from 0.2 µg/mL to 100 µg/mL. Tamoxifan was used as a controlled drug and dissolved in DMSO. The controlled culture cells received the same treatment for concentration of solvent. The final concentration of DMSO was 0.1% (v/v) or less. Each concentration of the extracts was assayed thrice. The cells were incubated at 37°C, 5% CO_2 in the incubator [13]. The assay was terminated after 24 hours. The numbers of surviving cells were determined by MTT staining assay. 50 µL of MTT solution (2 mg/mL MTT in plain culture medium) was added to each well after 4 hours of incubation. MTT assay was then removed. The purple formation crystal formed at the bottom of the wells was dissolved with 200 µL DMSO before being placed on the shaking microplate for 20 minutes. The effect of the samples on the proliferation of HepG2, MCF-7 and HT 29 cells were expressed as % cell viability, using the following formula:

$$\frac{(OD\ of\ drug\ treated\ sample - OD\ of\ blank)}{(OD\ of\ sample - OD\ of\ blank)}\ x\ 100$$

A dose-response curve was plotted to obtain the IC_{50} values. The IC_{50} is the concentration of extracts and fractions, in which growth was inhibited in 50% of the cancer cell population. All analytical values shown represent the means of triplicates. The data are expressed as mean ± standard deviation. Statistical significant difference was determined using One-way ANOVA and differences were considered to be significant if $p < 0.05$.

2.5 Identification of compounds

The structures of the compounds isolated from *C. nutans* were determined by one- and two-dimensional ^{1}H and ^{13}C nuclear magnetic resonance (NMR) experiments (HSQC, ^{1}H-^{1}H COSY and HMBC) and measured on a Bruker (Rheinstetten, Germany) DMX Spectrometer operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C nucleus. Isolates 1 and 2 were dissolved in deuterated chloroform and at room temperature.All spectra were obtained in trimethylsilane (TMS) with chemical shifts δ in ppm and coupling constant (J) in Hertz (Hz). High-resolution MS spectra were acquired in positive ion mode on a UPLC/Q-ToF-MS (Waters) equipped with an electrospray ion source (ESI) and time of flight analyser.

3. LITERATURE REVIEW

3.1 Botanical Overview

Clinacanthus nutans is one of the important species from family of Acanthaceae and has been used as a great herbal medicine in Asian Tropical. It has well known as Sabah snake Grass or snake plant or Belalai Gajah in Malaysia. [14] Table 2.1 described the taxamony of the *C. nutans*

Table 2.1 The taxamony of *C. nutans*

Scientific Classifications:	
Kingdom	Plantae
phylum	Magnoliophyta
class	Magnoliopsida
order	Lamiales
Family	Acanthaceae
Genus	*Clinacanthus*
Species	*Clinacanthus nutans*

Source: [9, 15]

3.2 Acanthaceae

C. nutans is one of the important species which came from this family. Acanthaceae is one of the leading of dicotyledonous flowering plant which includes 250 genus about 2500 species [16]. Moreover, Acanthaceae are classified into four subfamilies which are Nelsonioideae, Acanthoideae, Thumbergioideaea and Avicennioideae [17].

Most of these species usually can be found in Indonesia, Malaysia, Africa, Brazil and Central America. They were usually shrubs, vines or herbaceous (annual or perennial) plants, although a few are tropical tree species [17]. This kind of family can be grown in the most of the habitat comprising dense or open forest, bushes, damp fields and others.

A literature survey revealed that Acanthaceae family is considered as one of the largest source of medicinal plants providing effective traditional medicines against specific health impediments and those can be simply predictable morphologically by their simple, differing, decussate, whole leaves, zygomorph flowers and their superior ovary. [15, 17]

Acanthaceae are the most important is traditional medicinal from the leaves until the root, people usually used extensively for wounds. One of studies has been possess that one of the Acanthaceae species possesses antifungal, cytotoxicity, anti-inflammatory and so on. Other than tjat, Acanthaceae emines, Dyschoristethumbergiiflora, Lepidagathisscariosa and Thumbergiaalate (part of Acanthaceae family) has been used these plants for cough, skin diseases, wounds, eye infections, anti-diarrhea, edena, pneumonia and backache centuries ago. A part from that, paste of leaves of Barberiagrandicalyx (Acanthaceae) used for snake bites. Ash of leaves of Justiciabetonica, Acanthus pubescens and Justiciaflava is used for dry caough, anti-diarrhea, flu and ulcer [18]. This proved that Acanthaceae's family gives an important contribution exspecially in alternative traditional medicine areas.

3.3 Genus Clinacanthus

Genus of *Clinacanthus* comprising only two species which is are found in *C. nutans* (Burm. f.) Lindau and *Clinacanthus siamensis Bremek* [19] and are mostly found in South East Asia [20] and most of morphologies are similar to each other. However, several phytochemicals studies have been done but none on *C. siamensis*. These species have prominent roles as traditional medicine and have been claimed as medical plant in South East Asia especially common in Thailand.

Previous studied has been proposed to distinguish the different between the *C. nutans* and *C. siamensis* through their properties and characteristic and also applied in anti-herpes simplex virus [21]. Apart from that, this two species is quiet similar to *Andrographis paniculata* species which people claimed this kind of species is India Snake grass. All have similar growth habits and leaf appearance but are distinguished by the flowers, stems with different medicinal properties. [22]

C. siamensis is a medium sized perennial shrub grows up to 2m in height belonging to the family Acanthaceae. [23] Phytochemical and bioactivity investigations of *C. siamensis* have not been previously reported however, *C. siamensis* species has been used in Thailand as a traditional original medicine for the treatment of insect-bite, skin rashes. [24] and have potential to relieve painful swelling. [21]

Previous studied have been intense about the potential of *C. siamensis* on influenza virus infection by [25], *C. siamensis* has a protective effect against infection by certain influenza viruses. This is the first report to show that *C. siamensis* extract has an inhibitory activity on influenza virus NA, an in vitro anti-influenza virus activity and an in vivo protective effect

Another species from *Clinacanthus* genus is *C. nutans* species. *C. nutans* is also well known as *giro de flores, cocodriloflor, e zui hua* (Chinese): *yudunsou* (Japanese), *yōuduncho*

*(*Korea), *ki tajam, dandang gendis* (Indonesia), *belalai gajah, sabah snake grass* (Malaysia), *ki tajam* (Sunda), *saled pangpon tua mea, phaya yor, mon kai, phak lin khiat* (Thailand), *and manh cộng; lá cầm; bìm bịp;xurong khiI (Vietnamese).*

C. nutans (Burm.f.) Lindau (familyAcanthaceae) is a medicinal herb that is native to many tropical Asia countries and commonly can be found including Malaysia, Thailand, Indonesia and China [26]. *C. nutans* is a shrub of 1-3 m height with pubescent branches in apparent. Leaves are pale green, simple, opposite, narrowly elliptic oblong with acute apex size 2.5- 13.0 cm long and 0.5-1.5 cm wide [19]. It has been used as herbal medicine for treatment of herpes infection, insect, and snake-bites and allergic response. It also possesses anti-inflammatory, antimicrobial and anti-viral activity against herpes simplex virus (HSV) [27]

3.4 Clinacanthus Nutans

C. nutans is a scientific name of Sabah Snake Grass also called (Thai name: Phaya Yo or Phaya Plong Thong) (Malay name: Belalai Gajah) , is a small shrub native to tropical Asia which is traditionally used as herb to cure snake bite, due to the belief of its ability to neutralize poison from the snake venom. It grows well in tropical weather such as in Malaysia, Thailand and Vietnam. [28]

According to Tran (2014), *C. nutans* is the one of family in herbs which it erect about 3 meter tall and sometimes rambling shrubs. The sterms is slightly cylindrical and yellow in color when it dries. It grows on the low altitude area from 500 to 620 meters, especially under the scattered forests and bushes on the low altitude.[29]

The shape of the *C. nutans* leaf is lanceolate (2.5-13.0 cm along x 0.5-1.5 cm wide) with acute apex and pale green in color with short hair branches. Glandular trichrome and lithocyst components lie beneath the lower epidermis of the leaf. The leaf base are concate, abtuse rounded or truncate and often oblique [30].

Its flowers are dull red in color with green and yellow streaks based on the lower lip located on the top branches with two stamens on the flower inserted in the throat. The petiole is 3-15 milimeter long, and leaf blade lanceolate-ovate, lanccolate or linear-lanccolate. The leaves the calyx of flower about 1 cm long with grandular-pubescent. Corolla is dull red with green base, about 3.0-4.2 cm. the ovary is compressed into two cells and each cell has two ovules, the styles are filiftom and shrtly bidentate. Capsule is oblong basally wrapped into 4- seeded short stalk [16]. The botanical features of *C. nutans* is depicted in Figure 2.1

Figure 2.1 (a) Whole plant, (b) Simple lanceolate leaves and (c) Flower
Source: [17], [16] and [31]

3.5 Medical Usage

C. nutans was considered as one of the largest sources of medicinal plants providing effective traditional medicines against specific health impediments and its well known as Thai traditional medicine against viral disease to human being

Besides, previous reports claimed that *C. nutans (Burm. f.) Lindau* (Acanthaceae), is one of the medicinal plants that have been used to treat poisonous snake bites, but later showed

to be potential in antiviral properties. This plant is still used in many hospitals in Thailand for yhe treatment of herpes simplex virus [21] and herpes zoster virus [32, 33]

Apart from that, due to its medicinal properties it have ability to treat skin rashes, insect and snake bits, skin lesions caused by virus, fever and Dengue disease (Lau et al., 2014; Kunsorn et al., 2013; Goonasakaran,[29] It is commonly consumed in the form of herbal tea for the treatment of diabetes mellitus, fever, diarrhoea and dysuria. [14]

Leaves of *C. nutans* also possess as raw material or mixed with apple juice, sugarcane, or green tea and provide as fresh drink, consumedly. [16] Other than that, in previous studied report, citizen in Indonesia, they usually apply this plant by boiled of the fresh leaves with plain water and drink it for treatment of diabetes

C. nutans has been widely used from the leaves as anti-inflammatory agents for treatment of allergic responses or insect bites [34]. Another report claimed that this plant demonstrated a potential to be used as natural nutraceuticals for cancer prevention and treatment. In previous studies, *C. nutans* possesses a good result in anticancer and antiproliferative properties against a few cell lines [8]. The effectiveness of this plant has been not yet to be proven scientifically and more research needs to be carried out. But In Thailand, patients have claimed that they have recovered from cancer illness after consuming the leaves over a certain period of time. [35]

3.6 Phytochemicalsof Genus *Clinacanthus*

Medicinal plants contain secondary metabolite also defined as bioactive phytochemical that are produced to protect the plant. This metabolite very essential to plant growth and determine the nutritional quality of food, colour, taste, antioxidant and other antiviral activities [4]. Secondary metabolites are organic molecules that are not involved in the normal growth and development of an organism. Secondary metabolites can be considered as end product of primary metabolites and are categories in three main groups depend on their biosynthetic origin, which

ate terpenes, nitrogen-containing alkaloids and phenolic compound [17]. They also contain numerous natural products with interesting pharmacology activities.

There are several bioactive compounds in constituting of genus *Clinacantus* It has been phytochemicals and chemically investigated in previous studies. A series of flavonoids, steroids, triterpenoids, cerebrosides, glycoglycerol-lipids, glycerides, sulfur-containing glyceride were isolated from this plant (Fen and Rossa, 2014) According to Fong (2015) saponins, phenolic acids and tannins also has been reported as one of the member of bioactive compound that contribute in theses genus. [17]

In previous studies, Sakdarat (2009) reported the six C-glycosyl flavones shaftoside, isomollupentin 7-O-β-glucopyranoside, orientin, isoorientin, vitexin and isovitexin were isolated from the methanolic extract of *C. nutans* leaves in his research.[36] They possess important biological activities including antimicrobial activity (isoorientin, vitexin), hepatoprotective activity (isoorientin), and antioxidant activity (isovitexin) was reported by Teshima (1997)

Lupeol, β-sitosterol and stigmasterol [15] were isolated from a light pertolrum extract from the stern of *C. nutans* by chromatography. These compounds have been extensively studied since 1977. Compounds isolated fron *C. nutans* has been identified as the member of steroid [37]

Another compound has been investigated from *C. nutans* by (Tuntiwachwuttikul, Pootaeng-on, Phansa, & Taylor, 2004). A mixture of cerebrosides and a monoacylmonogalactosyl glycerol [(2S)-1-O-linolenoyl- 3-O-b-Dgalactopyranosylglycerol] were isolated from the EtOAc-soluble fraction of the ethanolic extract of the fresh leaves of *C. nutans* .

Based on previous report, eight compounds has been isolated from *C. nutans* leaves were related to chlorophyll a and chlorophyll b derivatives from chloroform extract of *C. nutans* leaves which are namely 132-hydroxy-(132S-) chlorophyll b, 132-hydroxy-(132R)-chlorophyll b, 132-hydroxy-(132S)-phaeophytin b, 132hydroxy-(132-R)-phaeophytin b, 132-hydroxy-(132S)-phaeophytin a, 132-hydroxy-(132-R)phaeophytin a, purpurin 18 phytyl ester and phaeophorbide a [38]. Five of these compounds were claimed as novel compounds. The studies were further investigating the potential of this compound for the determination of their anti-HSV-1 activity (Sakdarat, 2009).

According to [39], A series of sulphur-containing glucosides were isolated from the n-BuOH soluble portion of a methanolic extract of the stems and leaves of *C. nutans* species. Five sulfur-containing glucosides were identified and determined by spectroscopy techniques namely clinacoside A , clinacoside B, clinacoside C, clinacoside D and clinacoside E.

An investigation has been done by [9] by applied Liquid chromatography mass spectrometry equipped with an electrospray ionization source liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI/MS) to analysed several phenolic and fatty acids in ethanol and ethyl acetate extracts from *C. nutans* species. Ten of phenolic compound and fatty acids that has been detected from this instrument which are alpha tocopherol, sinapic acid, 4-hydrophenylacetic acid, 4-hydroxybenzoic acid, coumalic acid and quercetin hydrate from ethyl acetate extract in *C. nutans* . While ethanol extract demonstrated several compounds which are p-coumaric acid (31), vanilic acid, stearic acid and linoleic acid. Gallic acid and caffeic acid were identified from methanol extract of the leaf of *C. nutans* and has been reported in [17] study.

Apart from that, digalactosyl diglycerides and trigalactosyl were isolated from the leave extract and possess anti-herpes simplex virus effect report by [15]. Other than that, another

previous research has been reported and four new compounds were discovered from ethanolic extract of aerial part of *C. nutans*. Four new sulfur-containing compound and three known compound was isolated from this study which are clinamide A (38), clinamide B, clinamide C, entadamide A, entadamide C and trans-2-methylsulfinyl-2-propenol.

Despite from that, *C. siamensis BREM* species has their own bioactive compounds. No report have been claimed previously about phytochemical and bioactivity investigations. n-BuOH-soluble fraction of the ethanolic extract of the fresh leaves of *C. siamensis* demonstrated two new sulfur-containing compounds comprising, trans-3-methylsulfonyl-2-propenol and trans-3-methylsulfinyl-2-propenol have been isolated together with trans-3-methylthioacrylamide, entadamide A and this report were claimed by Tuntiwachwuttikul, Pootaeng-On, Pansa, Srisanpang and Taylor [24]. From previous report, some bioactive compounds exist in both species according to their characteristics and its potential. All the structures of the compounds were identified by spectroscopy data.

3.7 Bioactivites of *C. nutans*

Malaysian and other Asian countries are broadly used *C. nutans* as an alternative traditional medicine instead using modern medicine which contain highly chemical added nowadays. So, many researchers have been come out with studies to investigate more about *C. nutans* abilities. Besides, reports and preliminary studies, Malaysian species of *C. nutans* gives potential lead compounds in drug discovery. Thus, various biological activities has been done and demonstrated the potential as anti-microbial, antioxidant, anti- inflammatory and cytotoxicity.in previous study

3.7.1 Anti-inflammatory, analgesic, antibacterial and antimicrobial activities

There were a few Studied were investigated for anti-inflammatory and immune-modulatory activities of *C. nutans* species so far. [34] reported the anti-inflammatory activity

from ethanolic extract of the *C. nutans (Burm.f.) Lindau* and *Barleria lupulina Lindl* showed that both herb extracts may be attributed to their inhibitory effects on neutrophil functions other than neutrophil migration. However, further investigation to fully identify the biologically active ingredients and to define the underlying molecular mechanisms of the inhibitory effects of these extracts on neutrophil functional responsiveness is required.

80% ethanol extract of the *C. nutans* showed anti-inflammatory, anti-dengue virus and immune-modulating activity. In this study, some new chemical compound was found and tested on anti-inflammatory activity and anti-dengue virus activity; the extract had the strongest elastase release inhibitory effect at 68.33%. Moreover, this extract displayed moderate anti-dengue virus activity in the IC50 31.04 μg/mL. On the other hand, using high concentration of 80% ethanol extract (100 μg/mL) led to down-regulation of IFN-γ exhibiting immune-modulating activity. However, the new compounds isolated so far did not showed any targeted activity [40]

Apart from that, *Clinacanthus nutans* extracts have been claimed as one of the medicinal herb found in South East Asia used for skin infections.[16] From this report, *C. nutans* leaf was extracted with methanol demonstrated antibacterial effect against the strains of *Propionibacterium acnes*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus cereus* and *Escherichia coli*. *C. nutans* has indicated significant antibacterial effect against *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*). Thus, a further study is required to identify the antibacterial of bioactive compounds. [41]

Another studies had done by [42] on screening of antimicrobial of *C. nutans* extracts . In MIC assay, all the crude extracts and fractions showed inhibition against all tested microorganisms. Fraction 7 displayed the lowest MIC and MBC/MFC values against *B. cereus* and *C. albicans*.

3.7.2 Anti- Varicella- Zoster Virus (VZV) and Anti-Herpes Samplex Virus (HSV) Activities

Varicella- zoster virus (VZV) causes varicella (chickenpox) in a primary infection, generally in childhood [43]. Another report was investigated by [33] the effect of crude *extract C. nutans* has been demonstrated the antiviral activitiy against varicella-Zoster Virus.(VZV) with three different treatments. The results showed *C. nutans* had effect on VZV detected by DNA hybridization and plaque reduction assay method which means depending on concentration and method of treatment

C. nutans extract has been possesses for the clinical trial in treatment of herpes zoster infection. The studied report that, patients were divided 2 categorised which is the *C. nutans* extract - treated group and the placebo group. The investigation was observed five times daily for 7-14 days until the lesions were healed. Hence, along the medication process, the number of patients with lesion crusting within 3 days and with lesion healing within 7 days and 10 days were significantly greater in the *C. nutans* extract - treated group compared to the placebo group. Pain scores were reduced more rapidly in the *C. nutans* extract - treated group than in the placebo group. Thus, this showed that *C. nutans* are capable to be one of the alternative medicines in clinical treatment. Other than that, there were no side effects of the study medication.[32]

A previous study has been proposed on difference between *C. nutans* and *C. siamensis* by assessing pharmacognosy characteristics, molecular aspect and evaluation of their anti-herpes simplex virus (HSV) type 1 and type 2 activities. In this study, there are slightly different between these two species in bioactive compounds that content in the plants itself. Besides that, this two species have exhibits good antiviral activity against both HSV-1 and HSV-2 which means these plants have the ability to be a good antviral agent.[21]

3.7.3 Antioxidant Activity

Several investigations have been claimed for antioxidant activity for *C. nutans* According to [35], an investigation has been done on antioxidant activity of *C. nutans* against free radical-induced hemolysis. An ethanolic extract of dried leaves of *C. nutans* was used in this study and focussed on natural sources of antioxidants for the protection of the body from oxidative stress. The extract demonstrated a significant inhibition of peroxide production in rat macrophages stimulated by phorbol myristate acetate (PMA) and protected red blood cell against AAPH-induced hemolysis with an IC50 of 359.38±14.02 mg/mL. Thus, this researched were proved that the ethanolic extract of CN had an antioxidant activity and protective effect against freeradical-induced hemolysis.

A previous studied was reported on antioxidant properties of *C. nutans* was evaluated using DPPH, galvinoxyl, nitric oxide, and hydrogen peroxide based radical scavenging assays, whereas the tumoricidal effect was tested on HepG2, IMR32, NCL-H23, SNU-1, Hela, LS-174T, K562, Raji, and IMR32 cancer cells using MTT assay and that data showed that *C. nutans* in chloroform extract showed good antioxidant against DPPH and galvinoxyl radicals, but less effective in negating nitric oxide and hydrogen peroxide radicals. Chloroform extract exerted the highest antiproliferative effect on K-562 (91.28±0.03%) and Raji cell lines (88.97±1.07%) at 100 μg/mL and the other five cancer cell lines in a concentration-dependent manner, but not on IMR-32 cells was claimed by [8]

Instead of that, the antioxidant activity and protective effects of *C. nutans* extracts on plasmid DNA integrity in E. coli was done by [44]. The antioxidant activities of *C. nutans* are lower compared with green tea. The superoxide dismutase (SOD) activity and total phenolic contents of green tea are almost higher than that of *C. nutans* leaves, respectively. However, *C. nutans* leaf extract's retention of the integrity of super-coiled plasmid DNA under riboflavin photochemical treatment was shown to be better in comparison with extracts of green tea.

Antioxidant assays from DPPH, ORAC and ß-carotene bleaching (BCB) assay of all the extract from *C. nutans* had been reported in this study. DPPH activity for *C. nutans* extracts showed highest in the ethyl acetate and dichloromethane extracts (semi polar extracts) followed by ethanol and n-hexane extracts. A recent report by [8] also documented that a chloroform extract (semi-polar) of *C. nutans* leaves exhibited highest antioxidant activity in a DPPH assay. ORAC assay based on HAT is a considerable antioxidant capacity assay that is the most appropriate to measure in vitro and in vivo action In this study, a variation in antioxidant capacity with ORAC assays which is ethanol extract of *C. nutans* had highest antioxidant capacity (229.5 mMol TE/100 g extract) followed by ethyl acetate (181.6 mMol TE/100 g extract), dichloromethane (115.5 mMol TE/100 g extract) and n-hexane (114.3 mMol TE/100 g extract).

Another research has been proposed on antioxidant activity form the *C. nutans* extracts. Ethanol and ethyl acetate extracts demonstrated strongest antioxidant activity compared n-hexane and dichloromethane extract. In this study, 1, 1-diphenyl-1-picrylhydrazyl DPPH assay, oxygen radical absorbance capacity assay (ORAC) and β-carotene bleaching activity assay was proposed for the antioxidant activity. Thus, ethanol and ethyl acetate extracts of *C. nutans* is the one of new source of antioxidant in herbal medicines research. [9]

Other previous report, the antioxidant activity and protective effects of *C. nutans* on plasmid DNA integrity in E. coli has been performed by [44]. The antioxidant activities of *C. nutans* are lower compared with green tea. The superoxide dismutase (SOD) activity and total phenolic contents of green tea are almost higher than that of *C. nutans* leaves, respectively. However, *C. nutans* leaf extract's retention of the integrity of super-coiled plasmid DNA under riboflavin photochemical treatment was shown to be better in comparison with extracts of green tea.

3.7.4 Anticancer Activity

Cancer is a deadly disease and hunting millions of people around the globe [45]. Another research has been done by [42] on the evaluation on screening of cytotoxicity, antioxidant and antimicrobial activities of *C. nutans* extracts. Petroleum ether extracts demonstrated the strongest cytotoxic activity against HeLa and K-562 cells with IC50 of 18.0 and 20.0µg/mL, respectively.

[28] was focussed on the evaluation of bioactivity of *C. nutans* : anticancer and antioxidant activities in this study. The DPPH Scavenging Assay was prepared to evaluate the antioxidant activities. Moreover, four differences type of cells such as HepG2, NCI H460, MCF-7 and Hela was used to evaluate for anticancer activities using Solforhodamine B colorimetric Assay. The result showed that the compound from ethyl acetate extract which is sub-fraction F-III quite strong cytotoxicity when against HepG2 with IC_{50} value of 36.80 µg/mL but no effect on antioxidant activities. Therefore, more studies about anticancer should be explored to bioassay the activity of other extracts and each identified compound further.

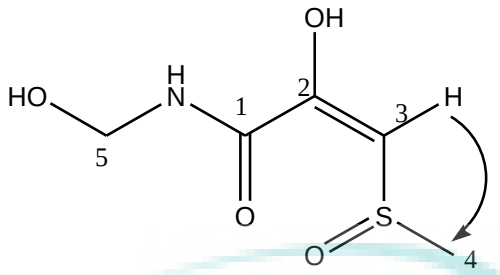
Previous study has been performed on the toxicity of the *C. nutans* plant. This study was carried out to evaluate the safety of methanol leaves extract of *C. nutans* by sub-acute 28-day (repeated doses) oral administration to male Sprague Dawley (SD) rat. The observation including changes in the body weight changed, water and food intake between the treatment group and control group. The observation showed 28 days oral administration methanol extract of *C. nutans* was safe in male SD rats without any side effects. [46]

4. FINDINGS

4.1 Isolation of active compounds and structural determination

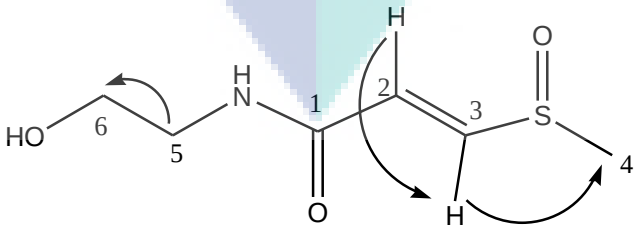
Two new sulphur-containing compounds (1) and (2) were isolated from the methanolic extracts of *C. nutans*. A comparison of the ^{1}H and ^{13}C NMR spectroscopic data of (1**)** and (2**)** (Table 1) with clinamide A and entanamide A (Tu et al., 2014) implied that they shared a similar skeleton, with a noticeable difference being in the presence of a methylsulfynil group for both skeletons instead of methylsulfonyl group.

Clinamide D (1) was assigned a molucelar formula based on molecular ion peak [M]+ at m/z 179.06266 at the HRESIMS corresponding to the molecular formula $C_5H_9NO_4S$.The ^{1}H NMR (500 MHz, $CDCl_3$) spectrum showed three singlet proton signals. The first singlet proton signals at the downfield region at δ_H 6.61 is attributed to one olefinic proton that is coupled with one hydroxyl group. The second singlet proton signals at δ_H 4.45 (H5) refers to one oxymethylene proton and the third proton signal at δ_H 2.63 is attributed to the methylsulfinyl group. The ^{13}C-NMR show four carbon signals, consisting of one methylsulfonyl group (δ_C 40.4), one oxymethylene group (δc 61.9), one methane group (δc 133.4) and one amide carbonyl group (δc 166.1). The full molecular structure of **1** was assembled based on homonuclear and long-range heteronuclear correlations from ^{1}H-^{1}H COSY and HMBC spectra. In HMBC spectrum only one correlation was found between H4 and C3. Furthermore, the hydroxyl group was assigned to C-2 since no cross peaks had been observed in COSY spectrum for olefinic proton. According to the interpretation, the structure (1**)** was established as clinamide D.



(1)

Clinamide E (2) was obtained as pale yellow oil and was assigned the molecular formula $C_6H_{11}NO_3S$, as deduced from HRESIMS at m/z 178.07767 $[M+1]^{+\cdot}$. The ^{1}H-NMR spectrum exhibited five proton signals. Two doublet signals at δ_H 6.72 (d, *J*=14.45) and 7.59 (d, *J*=14.45) were attributed to olefeinic protons in *trans*-relationship. An upfield signals appeared as triplet and multiplet at δ_H 3.59 (m) and 3.79 (t, *J*=5.05) were corresponding to to the methylene protons (Table 1). The sharp singlet at δ_H 2.71 corresponding to methylsulfinyl group. The structure of 2 was found similar to clinamide B [40]. The proton spectrum The ^{13}C-NMR showed six carbon signals, consisting of one methylsulfinyl group (δ_C 39.7), one oxymethylene group (δc 62.1), one methylene group (δc 42.7), two methine groups (δc 128.0 and 147.6), and one amide carbonyl group (δc 166.4). The COSY spectrum indicated two correlation between H5/H6 and H2,/H3. In HMBC spectrum only one correlation was found between H4 and C3. According to above interpretation, the structure (2) was established as clinamide E.



(2)

Table 1 ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data of compounds **1** and **2**

Position	1		2	
	δ_C (ppm)	δ_H (ppm) Int. Mult. J	δ_C (ppm)	δ_H (ppm) Int. Mult. J
1	166.1	-	166.4	-
2	n.o	-	128.0	6.72 (1H, d, 14.45)
3	133.4	6.61 (1H, s)	147.6	7.59 (1H, d, 14.45)
4	40.4	2.63 (3H, s)	39.7	2.71 (3H, s)
5	61.9	4.45 (2H, s)	42.7	3.59 (2H, m)
6			62.1	3.79 (2H, t, 5.05)

n.o-not observed

4.2 Antioxidant activity

Antioxidant activities of natural products could not be reasonably validated by a single method due to the complexity of natural products and the limitation of each antioxidant assay method. In the present study, two *in-vitro* antioxidant assays, DPPH scavenging and FRAP assays, were selected, which produced complementary results. DPPH and FRAP radical scavenging activities of different solvent extracts are shown in Table 2. $CHCl_3$ and MeOH extracts at 1 mg/ml concentration inhibited 70.96 % and 69.09 % of DPPH radical respectively; however, hexane and EtOAc extracts inhibited only less than 55% of DPPH radical. The results are in tandem with previous studies which indicate high antioxidant activity in DPPH assay from chloroform extract followed by methanol extract [8]. Isolated compounds 1 and 2 showed potent

radical scavenging against DPPH with 76.05% and 72.84% respectively. Our data show that chloroform extract was a good antioxidant against DPPH but less effective in FRAF assay.

Table 2

Antioxidant activity of C. *nutans* plant extracts

Assay	Standard*	Hexane	MeOH	$CHCl_3$	EtOAc	1	2	FB28
DPPH [a,b]	74.76 ± 0.05	50.50 ± 0.03	69.09 ± 0.04	70.96 ± 0.03	55.82± 0.01	76.05 ± 0.02	72.84 ± 0.01	71.9 ± 0.01
FRAP [a,b]	92.41 ± 0.05	-	56.49 ± 0.05	34.43 ± 0.05	-	15,47 ± 0.03	38.56 ± 0.02	55.7 ± 0.03

* Ascorbic acid.

[a] Mean values (n=3) with significant difference at P <0.05.

[b] Percentage of inhibition due to extract concentration of 1000 µg/ml

Reducing power, as a significant indicator of potential antioxidant activity, is the key index of antioxidant effects. The reducing power of a bioactive substance is closely related to its antioxidant ability. FRAP ability of MeOH extract was 56.49% and higher compared to $CHCl_3$ with 34.43 % . Hexane and EtOAc extracts do not show any inhibition activity through FRAP assay. In view of the potent antioxidant activity for both assays, the MeOH extract was chosen for further purification on the column chromatography.

A different IC_{50} value was observed for MeOH extract, fraction and isolated compounds (Table 2). The results of antioxidant activity, expressed as IC_{50}, indicate that compounds (1) show the highest DPPH radical scavenging activity and ferric reducing potential compared to other samples. This is due to the presence of two hydroxyl groups in the structure 1 compared to only one hydroxyl group in structure 2. Generally, antioxidant activity increases with an increase of hydroxyl group [47]. The presence of O-H groups may be responsible for antioxidant efficiency, similar to phenolic antioxidants, via chain-breaking mechanism of phenolic hydrogen. Compound (2) in this study shows the ability to quench the DDPH radical and reduces the ferric ions to ferrous ions but less potent compared to fraction FB28. Fraction FB28 show higher antioxidant capacity, because of t the mixture of sulphur compounds 1 and 2. Comparison between diallylsulfide and diallyldisulfide in garlic show that disulphide is more potent antioxidant compared to monosulfide [48]. The abundance of sulphur molecules in fraction FB28 may contribute to synergistic antioxidant property rather than individual sulfur- containing compounds (2).

Sulphur containing compounds isolated in this study may have a significant contribution to the antioxidant and anticancer properties of *C. nutans*. This is the first report of sulfur containing compound with antioxidant activity from *C. nutans*. Small molecules antioxidants containing sulfur can ameliorate oxidative damage and cells employ multiple mechanisms to prevent cellular damage [49]. The number of sulfur atoms determines their modulatory activities on the gluthathione related antioxidant enzymes[48]. Gluthathione plays a vital role in the cells to detoxify radicals and other electrophilic species, in particular with the help of gluthathione-S-

transferases (GSH) found in the cells [48]. Dietry sulfur-containing compounds may be used therapeutically as it is a precursor of GSH.

4.3 Anticancer Activity

The IC_{50} values of the effect of *C. nutans* methanol extract on the HepG2, MCF-7 and HT 29 cell lines after incubation at 24 h, 48 h and 72 h are summarized in Table 3. The results exhibit significant activity against cell lines under 72 h incubation with IC_{50} value 36.19 µg/mL on HT 29, Hep G2, 48.37 and MCF-7 54.16 µg/mL. Strong cytotoxic effect obtained from methanol extract against HT 29 followed by Hep G2 and MCF-7. Results of this study are in agreement with previous findings that reporting the crude methanol extract of C. *nutans* showed significant cytotoxicity against the D24 melanoma, but not normal cells and suggests that the extract is selective against cancer cells [50]. The present study also contributes to the evidence that the extract of *C. nutans* does indeed possess antiproliferative importance.

Cell lines	**IC_{50}(µg/mL)**		
	24hr	**48hr**	**72hr**

Table 3 IC_{50} values after 24, 48 and 72 hours of incubation of *C. nutans* extracts against HepG2, MCF-7 and HT 29 cancer cell lines.

MCF 7	ND	ND	54.164±0.991
Hep G2	84.080±0.461	94.825±1.511	48.370±0.026
HT 29	72.11±1.036	50.11±0.864	36.19±1.058

The IC_{50} values on different cell lines are, fall above the recommended IC_{50} value by National Cancer Institute of America, for crude extract, which < 30 µg/mL [42] . Although this data suggest that methanol extract from *C. nutans* may not be a strong anticancer regimen, cancer inhibitory properties showed in this experiment may still support the uses of *C. nutans* as an alternative adjunctive therapy for cancer prevention or treatment. The presence of glycosyl group in flavone ring may contribute to the lower cytotoxicity of effect of methanol *C. nutans* extracts. Glycoside has a higher solubility in water due to the hidrophilicity of the sugar moieties. The higher hydrophilicity of glycosyl residue caused them to be barely incorporate well into the cells [51] and therefore showed less effect in the present study. We assume the presence of two glycosyl group in the major compound corymboside would decrease the cytotoxicity effect; however, longer exposure duration would allow cells to uptake and digest the glucosyl residues.

5. CONCLUSION

Sulphur-containing compounds isolated in this research may provide scientific evidence for testimonies. Malaysian newspapers report that *C. nutans* possesses antitumor effects and has saved many lives suffering from various cancers. Sulfur-containing compounds could be a source of cytoprotective antioxidant based anticancer regimen. These findings suggest that *C. nutans* extracts could be used as an alternate chemopreventive regimen for cancer patients. For medicinal purposes, the safety and toxicity of this plant species still needs to be addressed.

ACHIEVEMENT

i) Name of articles/ manuscripts/ books published

This project produced two journal papers

Hamid, Hazrulrizawati Abd, et al. "Bioassay-guided Isolation and Antioxidant Activity of Sulfur-containing Compounds from Clinacanthus nutans." Journal of the Chinese Chemical Society (2016).

Hamid, Hazrulrizawati Abd, and Izzah Hayati Yahaya. "Cytotoxicity of Clinacanthus Nutans Extracts on Human Hepatoma (Hepg2) Cell Line." International Journal of Pharmacy and Pharmaceutical Sciences 8.10 (2016): 293-295.

ii) Title of Paper presentations (international/ local)

Antioxidant and antiproliferative properties of methanol extract of *Clinacanthus nutans* and identification of chemicals profile by UPLC-QTOF-MS. 2nd International Symposium on Phytochemicals in Medicine and Food (2-ISPMF) to be held in Fuzhou, China from April 7-10, 2017. (Abstract accepted)

iii) Human Capital Development

The following student works for this project

Master student

- Izzah Hayati Yahya (MKD14007) –in progress of writing thesis

Undergraduate student

- Fatin Nur Nazhatul Shahiraeh Binti Azmi (SA12010) - graduated November 2016
- Nurul Nadia Bt Salleh (SA12014) - graduated November 2016

iv) Awards/ Others

Chewable CliNu, Sulfur Health. Creation, Innovation, Technology & Research Exposition (CITREX), 7-8 March 2016, UMP- Bronze Medal

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APPENDIX A: JOURNAL

Article

JOURNAL OF THE CHINESE CHEMICAL SOCIETY

Bioassay-guided Isolation and Antioxidant Activity of Sulfur-containing Compounds from *Clinacanthus nutans*

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(Received: September 26, 2016; Accepted: October 20, 2016; DOI: 10.1002/jccs.201600755)

Clinacanthus nutans has been used in traditional herbal medicine for cancer prevention, but the specific bioactive compounds responsible for the observed activities have not been explored. Different polar solvents such as methanol, chloroform, ethyl acetate, and hexane were used for the extraction. The extracts, fractions, and isolated compounds were subjected to DPPH and ferric reducing antioxidant potential (FRAP) assays. Methanol extracts show significant free-radical scavenging activity of 69.09% in DPPH and 56.49% FRAP. Purification of MeOH extracts afforded the fraction FB28 and two new sulfur-containing compounds, named clinamide D and E (**1**, **2**). Compound (**1**) proved to be more active with an IC_{50} value for DPPH radical scavenging of 118.27 ± 0.01 μg/mL and reduction of Fe^{3+}–TPTZ complex of 386.24 ± 0.02, higher than that of the standard ascorbic acid. Sulfur-containing compounds isolated from *C. nutans* is a potential natural antioxidant.

Keywords: *Clinacanthus nutans*; Sulfur-containing compounds; Antioxidant; Belalai gajah.

INTRODUCTION

Oxidative stress, which is a basic pathogenesis of human ailments, results from the imbalance between the generation of free radicals and the scavenger antioxidant system.[1] Oxidative stress can be stifled by dietary cancer-prevention agents, which highlights the component of the victualing routine as a wellspring of rummaging free radicals that accommodate to diminish the risk of oxidative damage. Much of what is known regarding the mechanism of the reduction of oxidative stress is associated with the activation of antioxidant replication through scavenging free radicals or preventing the generation of free radicals, which presents a subsidiary target for health promotion.[2]

With many well-known scientists actively studying antioxidants as protecting agents, research on the effects of antioxidants on cancer susceptibility, molecular interactions, and overall health has become very intense.[3] Focusing our attention on natural sources of antioxidants for the protection of the body from oxidative stress, we have investigated the antioxidant activity of *Clinacanthus nutans*.

C. nutans has been traditionally used in different regions of Asia because of its many different pharmacological effects. Usually, the fresh leaves are boiled with water and consumed as herbal tea in Malaysia. *C. nutans* has long been used in Thailand as a traditional medicine for the treatment of skin rashes, insect and snakebites, herpes simplex virus (HSV), and varicella-zoster virus (VZV) lesions.[4] The leaves are consumed raw or mixed with apple juice, sugarcane, or green tea as a beverage.

C. nutans is a small shrub, native to tropical Asia. This plant is popular among Malaysians for its high medicinal value in treating cancer.[5] Although a few studies have been conducted related to the antioxidant properties of *C. nutans* exract,[6–8] there are no report on the isolated compounds exhibiting antioxidant activity. In our continuing efforts to find new natural antioxidant compounds, different extracts of *C.nutans* were investigated in order to isolate and identify its constituents with antioxidant activity and to provide complementary evidence for the presence of these phytochemicals that influence the antioxidant property of *C. nutans*.

RESULTS AND DISCUSSION

Isolation of active compounds and structural determination

Two new sulfur-containing compounds, (**1**) and (**2**), were isolated from the methanolic extracts of *C. nutans*. A comparison of the ^{1}H and ^{13}C NMR spectroscopic data of (**1**) and (**2**) (Table 1) with clinamide A and entanamide A (Tu *et al.*[9]) suggested that they

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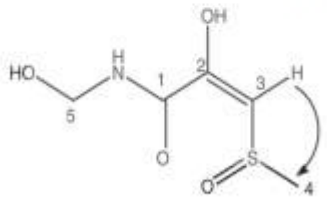
Table 1. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data of compounds **1** and **2**

	1		**2**	
Position	δ_C (ppm)	δ_H (ppm) *Int. Mult. J.*	δ_C (ppm)	δ_H (ppm) *Int. Mult. J.*
1	166.1	—	166.4	—
2	n.o.	—	128.0	6.72 (1H, d, 14.45)
3	133.4	6.61 (1H, s)	147.6	7.59 (1H, d, 14.45)
4	40.4	2.63 (3H, s)	39.7	2.71 (3H, s)
5	61.9	4.45 (2H, s)	42.7	3.59 (2H, m)
6			62.1	3.79 (2H, t, 5.05)

n.o., not observed.

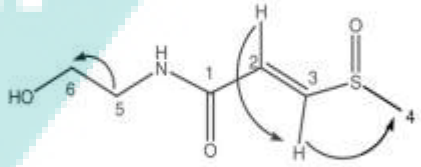
shared a similar skeleton, with the noticeable difference being in the presence of a methylsulfynil group for both skeletons instead of a methylsulfonyl group.

Clinamide D (**1**) was assigned the molecular formula $C_5H_9NO_4S$, based on molecular ion peak [M] + at *m/z* 179.06266 in the high-resolution electrospray ionization (HRESI) mass spectra. The ^{1}H NMR (500 MHz, $CDCl_3$) spectrum showed three singlet proton signals. The first singlet proton signal at the downfield region at δ_H 6.61 is attributed to one olefinic proton that is coupled to one hydroxyl group. The second singlet proton signal at δ_H 4.45 (H5) refers to one oxymethylene proton, and the third proton signal at δ_H 2.63 is attributed to the methylsulfinyl group. The ^{13}C-NMR spectrum shows four carbon signals, consisting of one methylsulfonyl group (δ_C 40.4), one oxymethylene group (δc 61.9), one methane group (δc 133.4), and one amide carbonyl group (δc 166.1). The full molecular structure of **1** was assembled based on homonuclear and long-range heteronuclear correlations from ^{1}H-^{1}H COSY and HMBC spectra. In the HMBC spectrum, only one correlation was found between H4 and C3. Furthermore, the hydroxyl group was assigned to C-2 because no cross peaks were observed in COSY spectrum for the olefinic proton. According to the above interpretation, compound (**1**) was established as clinamide D.



(**1**)

Clinamide E (**2**) was obtained as a pale yellow oil and was assigned the molecular formula $C_6H_{11}NO_3S$, as deduced from the HRESI MS peak at *m/z* 178.07767 $[M + 1]^+$. The ^{1}H-NMR spectrum showed five proton signals. The two doublets at δ_H 6.72 (d, J = 14.45) and 7.59 (d, J = 14.45) were attributed to olefeinic protons in trans relationship. The upfield signals appearing as a triplet and a multiplet at δ_H 3.59 (m) and 3.79 (t, J = 5.05) corresponded to the methylene protons (Table 1). The sharp singlet at δ_H 2.71 corresponded to the methylsulfinyl group. The structure of **2** was found similar to that of clinamide B.[9] The ^{13}C-NMR spectrum showed six carbon signals, consisting of one methylsulfinyl group (δ_C 39.7), one oxymethylene group (δ_C 62.1), one methylene group (δ_C 42.7), two methine groups (δ_C 128.0 and 147.6), and one amide carbonyl group (δ_C 166.4). The COSY spectrum indicated two correlations between H5/H6 and H2,/H3. In the HMBC spectrum, only one correlation was found between H4 and C3. According to above interpretation, compound (**2**) was established as clinamide E.



(**2**)

Antioxidant activity

The antioxidant activities of natural products cannot be reasonably validated by a single method due to their complexity and the limitation of each antioxidant assay. In the present study, two *in vitro* antioxidant

Table 2. Antioxidant activity of *C. nutans* plant extracts

Assay	Standard[a]	Hexane	MeOH	$CHCl_3$	EtOAc	1	2	FB28
DPPH[b,c]	74.76 ± 0.05	50.50 ± 0.03	69.09 ± 0.04	70.96 ± 0.03	55.82 ± 0.01	76.05 ± 0.02	72.84 ± 0.01	71.9 ± 0.01
FRAP[b,c]	92.41 ± 0.05	—	56.49 ± 0.05	34.43 ± 0.05	—	15.47 ± 0.03	38.56 ± 0.02	55.7 ± 0.03

[a] Ascorbic acid.
[b] Mean values ($n = 3$) with significant difference at $p < 0.05$.
[c] Percentage of inhibition due to extract concentration of 1000 μg/mL.

assays, DPPH scavenging and FRAP (ferric reducing antioxidant potential), were selected, which give complementary results. DPPH and FRAP radical scavenging activities of different solvent extracts are shown in Table 2. $CHCl_3$ and MeOH extracts at 1 mg/mL concentration inhibited 70.96 and 69.09% of DPPH radicals, respectively; however, the hexane and EtOAc extracts inhibited only <55% of DPPH radicals. The results are in tandem with previous studies that indicate high antioxidant activity in DPPH assay from the chloroform extract followed by methanol extract.[6] The isolated compounds **1** and **2** showed potent radical scavenging against DPPH with 76.05 and 72.84%, respectively. Our data show that the chloroform extract was a good antioxidant against DPPH but less effective in FRAF assay.

Reducing power, as a significant indicator of potential antioxidant activity, is the key index of antioxidant effects. The reducing power of a bioactive substance is closely related to its antioxidant ability. FRAP of MeOH extract was 56.49%, which is higher than that of $CHCl_3$ (34.43%). Hexane and EtOAc extracts did not show any inhibition activity in the FRAP assay. In view of the potent antioxidant activity for both assays, the MeOH extract was chosen for further purification on column chromatography.

A different IC_{50} value was observed for the MeOH extract, fraction, and isolated compounds (Table 2). The results of antioxidant activity, expressed as IC_{50}, indicate that compound (**1**) has the highest DPPH radical scavenging activity and ferric reducing potential compared to the other samples. This is due to the presence of two hydroxyl groups in the structure of **1** compared to only one hydroxyl group in the structure of **2**. Generally, antioxidant activity increases with of the number of hydroxyl groups.[10] The presence of O–H groups may be responsible for the antioxidant efficiency, similar to phenolic antioxidants, via the chain-breaking mechanism of phenolic hydrogen. Compound (**2**) in this study shows the ability to quench the DDPH radicals and reduces the ferric ions to ferrous ions, but it is but less potent than fraction FB28. Fraction FB28 shows higher antioxidant capacity because of the mixture of sulfur compounds **1** and **2**. Comparison between the diallylsulfide and diallyldisulfide in garlic shows that the disulfide is a more potent antioxidant than the monosulfide.[11] The abundance of sulfur molecules in the fraction FB28 may contribute to the synergistic antioxidant property rather than individual sulfur-containing compounds (**2**).

Sulfur-containing compounds isolated in this study may have a significant contribution to the antioxidant and anticancer properties of *C. nutans*. This is the first report of sulpfur-containing compounds with antioxidant activity from *C. nutans*. Small-molecule antioxidants containing sulfur can ameliorate oxidative damage, and cells employ multiple mechanisms to prevent cellular damage.[12] The number of sulfur atoms determines their modulatory activities on the glutathione-related antioxidant enzymes.[11] Glutathione plays a vital role in the cells to detoxify radicals and other electrophilic species, in particular with the help of glutathione-*S*-transferases (GSHs) found in the cells.[11] Dietary sulfur-containing compounds may be used therapeutically, as they are precursors of GSH.

EXPERIMENTAL

Chemicals

The compounds 2,2-diphenyl-1-picryl-hydrazyl (DPPH), and ascorbic acid as standard were purchased from Sigma Aldrich (Singapore). Silica gel $60F_{254}$ and precoated silica gel aluminium plates F_{254} were purchased from Merck (Darmstadt, Germany). Solvents used for chromatography and purification were purchased as analytical grade reagents from Merck.

Plant materials

The whole plant of *C. nutans* was collected in Jelebu, Negeri Sembilan, Malaysia. The plant was authenticated by Dr, Shamsul Kamis, Institute of Bioscience, Universiti Putra Malaysia (UPM). A voucher specimen number (SK 2874/15) has been deposited at the Herbarium Unit of UPM.

Extraction and isolation

Powdered and dried *C. nutans* (5.0 kg) were defatted by percolation with hexane and filtration. The plant material was dried, extracted through three cycles of sonication (30 min each) with MeOH, filtered to obtain a dark green extract, and was concentrated under low pressure. The concentrated extract was partitioned with $CHCl_3$. The $CHCl_3$ phase was collected and concentrated, leaving a dark green residue. The aqueous phase was partitioned with EtOAc to yield a dark green residue. The MeOH extract (18.311 g) was chromatographed using a column with silica gel slurry packed in $CHCl_3$. Elution was initiated with $CHCl_3$, with progressively increasing concentrations of MeOH in $CHCl_3$, until it reached 100% MeOH. This protocol yielded 21 subfractions (F1–F21). F8 (2.31 g) (664.5 mg) was chromatographed with $CHCl_3$/MeOH (60:40) as eluting solvent to afford 21 subfractions (FA1–FA21). Subfraction FA5 (128.5 mg), which was re-chromatographed with $CHCl_3$–MeOH (80:20) yielded compound **(1)** (1.71 mg). Subfraction FA9 was chromatographed with $CHCl_3$–MeOH (85:15) to afford subfraction FB28 and compound **(2)** (24.7 mg).

DPPH free-radical scavenging assay

The potential antioxidant activities of *C. nutans* extracts and the isolated compounds were determined using the method described by Foon *et al.*[13] with some modifications. A stock solution of the plant extracts and isolated compounds were prepared in different aliquots (31.25, 62.5, 125, 250, 500, and 1000 µg/mL), and each of them (100 µL) was mixed with 100 µL of DPPH methanolic solution (0.16 mM) in a 96-well plate. The mixtures were swirled gently for 1 min and kept in a dark room for 30 min. The absorbance of the samples was read at 540 nm in triplicate using the Tecan Infinite M200 PRO microplate reader at 540 nm. Butylhydroxytoluene (BHT) was used as a reference compound in this assay. The ability of scavenging the DPPH radical was calculated with the following equation: % inhibition = $[(A_{control} - A_{sample})/A_{control}] \times 100$.

Ferric reducing antioxidant potential assay

FRAP assay was conducted according to the method described by Benzie and Strain[14] with some modifications. The FRAP reagent was freshly prepared with 30 mmol/L acetate buffer (pH3.6), 10 mmol/L 2,4,6-Tri(2-pyridyl)-1,3,5-triazine, 40 mmol/L HCl and 20 mmol/L $FeCl_3$, 20 mmol/L $FeCl_3.6H_2O$. Working FRAP reagent was prepared by mixing acetate buffer, TPTZ solution, and $FeCl_3.6H_2O$ to the ratio of 10:1:1. This method is based on the reduction of ferric 2,4,6-tri (2-pyridyl)-1,3,5-triazine complex (Fe^{3+}-TPTZ) to its ferrous (Fe^{3+}-TPTZ) form with intense blue color in the presence of the antioxidant. The reagent solution was warmed to 37°C before use. Twenty microliters of *C. nutans* extract and the isolated compound were mixed with 200 µL of the reagent into 96-well plates. The mixture was left for 10 min and the absorbance was read at 593 nm using a Tecan Infinite M200 PRO microplate reader. Ascorbic acid was used as control. The ability of the samples to scavenge free radicals was calculated and was reported as % scavenging rate = $(1 - A_{sample}/A_{control}) \times 100$.

Identification of compounds

The structures of the compounds isolated from *C. nutans* were determined by one- and two-dimensional ^1H and ^{13}C nuclear magnetic resonance (NMR) experiments (HSQC, ^1H-^1H COSY, and HMBC) measured on a Bruker (Rheinstetten, Germany) DMX Spectrometer operating at 500 MHz for ^1H and 125 MHz for ^{13}C nuclei. Isolates **1** and **2** were dissolved in deuterated chloroform at room temperature. All spectra were obtained in trimethylsilane (TMS) with chemical shifts δ in ppm and coupling constant (*J*) in hertz (Hz). High-resolution MS spectra were acquired in positive-ion mode on a UPLC/Q-ToF-MS instrument equipped with an ESI source and a time-of-flight analyzer.

CONCLUSIONS

Sulfur-containing compounds isolated in this work may provide scientific evidence for their curative testimonies. Malaysian newspapers report that *C. nutans* possesses antitumor effects and has saved many lives from various cancers. Sulfur-containing compounds could be a source

of cytoprotective antioxidant-based anticancer therapy. These findings suggest that *C. nutans* extracts could be used as an alternate chemopreventive regimen for cancer patients. For medicinal purposes, the safety and toxicity of this plant species still needs to be addressed.

ACKNOWLEDGMENTS

The authors would like to thank the Ministry of Higher Education, Malaysia, for their support and a research grant RDU140102 to HAH.

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International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 8, Issue 10, 2016

Short Communication

CYTOTOXICITY OF *CLINACANTHUS NUTANS* EXTRACTS ON HUMAN HEPATOMA (HepG2) CELL LINE

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Received: 23 Jun 2016 Revised and Accepted: 12 Aug 2016

ABSTRACT

Objective: *Clinacanthus nutans* is one of the herbs that has been used in Asia as a traditional medicine for the treatment of serious diseases. The aim of this study is to investigate the phytochemical constituents (flavonoids and phenolics) and cytotoxicity against human hepatoma (HepG2) cancer cell lines of *C. nutans* extracts.

Methods: The fractions from *C. nutans* were extracted from hexane, methanol, chloroform and ethyl acetate by the solvent-solvent extraction method. The crude extracts (10 mg/ml) were tested against HepG2 cell lines using 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Phytochemical screening was done to determine the total phenolic content (TPC) and total flavonoid content (TFC).

Results: Methanol extract showed the strongest cytotoxic activity against HepG2 cell line with IC_{50} of 43.9367µg/ml after 24 h of treatment compared to chloroform extract and ethyl acetate, 55.6112µg/ml and 62.0655µg/ml, respectively. Hexane extract formed the lowest cytotoxicity activity with IC_{50} of 68.3807µg/ml. Total phenolic content (TPC) was found to be highest in chloroform, which was 119.29 mg of gallic acid equivalent (GAE) and total flavonoid content (TFC), methanol was performed the highest value, which is 937.67 mg of butylated hydroxytoluene (BHT).

Conclusion: Different active compounds present in the extracts may contribute different cytotoxicity effects of crude extracts. The relationship data of total phenolic, total flavonoid, and cytotoxic potential of *C. nutans*, indicates that these plants might contain valuable active compounds as a chemotherapeutic agent. Further investigations to elucidate the chemical structures of active compounds are necessary for potential compounds discovery in drugs.

Keywords: *Clinacanthus nutans*, Cytotoxicity, Human hepatoma (HepG2) cell line

DOI: http://dx.doi.org/10.22159/ijpps.2016v8i10.13626

INTRODUCTION

Cancer is the most distressing and life-threatening disease that enforces severe death worldwide [1]. Moreover, Cancer remains as one of the major health threats to Malaysians with a yearly mortality rate of cancer patients that has consistently reached 10-11% [2]. Current treatments including radiotherapy and chemotherapy are mostly ineffective against advanced stages of cancer and associated with severe side effects. [1] In addition, these treatments are not selective of cancerous cells and their therapeutic efficiency is limited due to the damage they can cause to healthy cells and tissues. To avoid these side effects in cancer therapy, there is an urgent need to develop therapeutic modalities with no or minimal side effects to normal organs. Use of plants for therapy is not new; indeed plants have been considered a valuable source of bioactive compounds for the treatment of many conditions, including cancer, in almost all cultures and communities for thousands of years [3].

Nowadays, there is a lot of research being done on the importance of medicinal plants for healing process compared to the existence of medicinal synthesize from the chemical. Herbs are one of in the types of plants, and some herbs have potential to cure serious diseases. In Indonesia, where herbal medicine is popular, more than 1300 species are known as medicinal plants, called Jamu [4]. These traditional herbal medicines still play an important role in the treatment of illnesses. *C. nutans* is one of the herbs that has been used in Thailand as a traditional medicine for the treatment of serious diseases [5]. *C. nutans* belongs to the family of Acanthaceae, which are known to have medicinal properties. This plant can be found in Malaysia, Thailand and Vietnam [6].

Previous phytochemical studies from *C. nutans* led to the isolation of flavonoids, steroids, triterpenoids, cerebrosides, glycol glycerol-lipids, glycerides, sulfur-containing glyceride from this plant [5, 7]. *C. nutans* has a viral potential for treatment of skin rashes, insect, and snakebite, herpes simplex virus (HSV), and varicella-zoster virus (VZ) [7, 8]. Moreover, previous studies reported that the chloroform extract from *C. nutans* are good in antioxidant against α-diphenyl–β-dipicryl–hydrazyl (DPPH) and galvinoxyl radicals and exert a high antiproliferative effect on human leukemia (K-562) cell line [2]. Although other researchers, but none of the reports has reported cytotoxicity of C. nutans in different doses, time and solvent extraction was tested on human hepatoma (HepG2) cell line. Apart from these reports, the correlation between cytotoxicity and phytochemicals contents may provide potential lead compounds in drug discovery. Thus, the aim of this work is to screen the total phenolic content (TPC), total flavonoid content (TFC) of the four different extracts and cytotoxicity toward human hepatoma (HepG2) cell line.

All solvents used were of analytical grade and included: methanol,chloroform, ethyl acetate, and hexane (Merck, Darmstadt, Germany). Folin–Ciocalteu phenol reagent, gallic acid, butylated hydroxytoluene, Roswell Park Memorial Institute medium (RPMI), fetal bovine serum (FBS), penicillin and streptomycin were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Ultraviolet (UV) spectra were recorded on a microplate reader (Tecan Infinite 200 Pro).

The dried powder was percolated with hexane (Fraction I) followed by methanol for a day. Both extracted plant samples were sonicated in 30 min over three cycles. Hexane (Fraction I) and methanol extraction (Fraction II) were filtered. The filtrates were evaporated to dryness using a rotary evaporator at a temperature below than 60 °C and with low pressure. Next, methanol extract was mixed with water in a ratio 3:1 and activated charcoal is a function to remove excessive chlorophyll before fractionate by dissolving the mixture with chloroform producing chloroform phase (Fraction III) and an aqueous phase, thrice. Then, ethyl acetate was added into the aqueous phase, thrice to obtained ethyl acetate phase (Fraction IV) and an aqueous phase. The extracts were concentrated in vacuo and evaporated using a rotary evaporator to get crude extractions.

TPC of C. nutans extract was determined by a method developed by [9]. A stock solution of plant extracts was prepared in methanol and pipetted out into test tube and mixed with 0.5 ml of Folin–Ciocalteu phenol reagent was added (FC reagent was dissolved in distilled water with 1:1 ratio). Then 2.5 ml of 20% sodium carbonate was added in each tube and finally the mixture was mixed properly by using vortex and then the test tube was kept in the dark for 40 min. Absorbance spectra was recorded at 725 nm using glass cuvettes. To minimize standard error, the reaction was tested thrice and the results were expressed in milligrams of gallic acid equivalent (mg GAE).

Total flavonoid content of plant extracts was determined by [9]. Concisely, 100 µl of each plant extract (1 mg/ml) was dissolved in corresponding solvents and then the extracts were made up to 1 ml by using distilled water followed by the addition of 75 µl of 10% sodium nitrate solution. After a 6 min interval, 150 µl of 5%aluminium chloride solution was added, and then 0.5 ml of 1 M NaOH was added in the test tubes. The mixture samples were added up to 2.5 ml by using distilled water and thoroughly mixed. The UV–V absorbance values were read immediately at 510 nm. The results were expressed in mg/g butylated hydroxytoluene (BHT) equivalents. Four different extracts, Fraction I, Fraction II, Fraction III and Fraction IV were tested on human liver hepatocellular carcinoma (HepG2) for their anticancer activity. The cell lines were stored in Roswell Park Memorial Institute medium (RPMI) medium containing 10% (v/v) fetal bovine serum (FBS) supplemented with penicillin (100 U/ml) and streptomycin (100 µg/ml) under humidified atmosphere containing 5% CO_2 in the incubator.

1 mg of four extracts was dissolved in 1 ml of 0.1 % of DMSO (with distilled water) as a stock solution of 0.1 mg/ml respectively. The testing solutions were made by half series dilution from 0µg/ml to 100µg/ml (0, 6.25, 12.5, 25, 50, 100µg/ml). These crude extracts were used for MTT assay

For the MTT assays cells (10 µl) were added into all the wells (104–105 cells per well) with various concentration of extracts and incubated in a 37 °C, CO_2 incubator for 24 h. A stock solution of 10 mg/ml MTT was prepared in PBS and MTT reagent (20 µl) was added to the cell monolayer. During this period, the living cells produced insoluble blue formazan from yellow soluble MTT. The reaction was stopped by the addition of dimethyl sulfoxide (DMSO, 100 µl) in each well. Control cells were incubated without the extract and with DMSO. The absorbance of samples was read at 540 nm using microplate reader.

Experiments were performed thrice. Results were expressed as percentage growth inhabitation of control. The percentage of inhibition of cell lines was calculated as shown below:

$$\%\ Inhibition = 100 - [\frac{Abs_{sample} - Abs_{blank}}{Abs_{control} - Abs_{blank}}] X\ 100$$

The IC_{50} values for each of the active extracts were determined by plotting the percentage inhibition values against the concentration of the extracts. A dose-response curve was used to enable the calculation of the concentrations that killed 50% of the HepG2 cells (IC_{50}). The results were expressed as mean±SD. One-way ANOVA was determined the concentration of extracts against the percentage of growth inhibition

Healthy HepG2 cells that achieved confluence were used in this assay. HepG2 cells exhibited epithelial-like structure to the bottom of the flask. The percentage inhibition of *C. nutans* extracts on HepG2 cell line after 24h of treatment as shown in table 1. The data shows that methanol extract performed the highest percentage of inhibition (74.17 %) against hepG2 cell line followed by ethyl acetate extract (69.67%), chloroform extract (64.80%) and hexane extract (61.67%) at a concentration of 100 µg/ml.

Table 1: Percentage inhibition of *Clinacanthus nutans* extracts on HepG2 cell line after 24h of treatment

Extracts	% inhibition at different concentration (µg/ml)				
	6.25	12.5	25	50	100
Hexane	11.67±0.24	20.00±0.32	42.50±0.83	43.75±0.06	61.67±0.75
Methanol	10.58±0.27	24.17±0.39	47.63±0.68	60.00±0.81	74.17±0.50
Chloroform	10.23±0.75	28.10±0.45	34.92±0.60	54.16±0.75	64.80±0.05
Ethyl Acetate	10.10±0.57	22.86±0.38	39.67±0.73	48.46±0.91	69.67±0.44

n=3; Values have been expressed as mean±standard deviation

The evaluation of cytotoxicity was done using the dose-response curve obtained by non-linear regression analysis. The IC_{50} values of all extracts are summarized in table 2. The percentage of inhibition of HepG2 cells is directly proportional as the extracts concentration increases. Methanol extract exhibited higher cytotoxicity on HepG2 cells after 24 h. The results obtained shows that methanol extract exhibited the strongest cytotoxicity activity against HepG2 cells line after a 24 hr treatment with IC50 of 43.9367 µg/ml followed by chloroform extract and ethyl acetate, 55.6112 µg/ml and 62.0655 µg/ml, respectively. Hexane extract performed the lowest cytotoxicity activity with IC50 of 68.3807 µg/ml.

Table 2: IC_{50} values of *Clinacanthus nutans* crude extracts against HepG2 lines at 24 h incubation

Cell line	Extracts	IC_{50} values (µg/ml)
HepG2	Hexane	68.38±0.07
	Methanol	43.93±0.05
	Chloroform	55.61±0.03
	Ethyl Acetate	62.06±0.06

n=3; Values have been expressed as mean±standard deviation

Table 3: Total phenolic content (TPC) and total flavonoid content (TFC) of C. nutans from four different type of solvent

Solvent extracts	Total phenolic content (mg/GAE)	TotalFlavonoid content (mg/g)
Standard	250.57±0.07*	533.22±0.04#
Hexane	28.25±0.02	586.67±0.01
Ethyl Acetate	22.17±0.02	532.33±0.02
Chloroform	119.29±0.07	428.67±0.03
Methanol	53.91±0.03	937.67±0.02

n=3; Values have been expressed as mean±standard deviation, *Gallic acid# α-BHT

Total phenolic content (TPC) and total flavonoid content (TFC)

The total of phenolic content (TPC) was found to be highest in the chloroform extract, which is 119.29 mg of GAE/g compared to other extracts. Total of flavonoid content (TFC), methanol crude performed the highest value, which is 937.67 mg of BHT/g compared with hexane and Ethyl Acetate, which are 586.67±0.001 mg/g and 532.33±0.0002 mg/g respectively. This chloroform extract had the lowest value (428.67±0.003 mg/g) (refer table 3)

The methanol extract from *C. nutans* showed the highest cytotoxicity effect compared to hexane, chloroform, and ethyl acetate extracts. Possibly, the activity might be due to the total flavonoid content which are highest in methanol extract. Methanol extracts treated with HepG2 cells, the IC_{50} values was 43.9367 µg/ml, which was above the recommended IC_{50} value by National Cancer Institute of America, for crude extract, which is<30 µg/ml [10]. Although this data suggests that methanol extract from *C. nutans* may not be a strong anticancer regimen, cancer inhibitory properties shown in this experiment may still support the use of *C. nutans* as an alternative adjunctive therapy for cancer prevention or treatment.

A similar study was reported by [6] that methanol extract from *C. nutans* yield sub-fraction F-III. Sub-fraction F-III inhibited the growth of all 4 cell line HepG2, NCI H460, MCF-7 and Hela. Subfraction F-III got the highest cytotoxicity with IC_{50} of 36.80µg/ml against HepG2 cell line compared to other cell lines.

In previous studies, Muhammad (2014) [5] claimed that six known C-glycosyl flavones were found in methanol extract that has been isolated earlier in the study. Flavonoid is a sub-class of phenolic and known with its polyphenolic structures. Flavonoids play an important role in protecting biological systems against harmful effects of oxidative processes on macromolecules [11]. Apart from the ability of a substance to modulate several biological activities, its direct interaction with cells *in vivo* can result in toxic effects [12].

Different flavonoids and phenolic compounds react with free radicals to reduce the degradation of membranes by preventing the reaction between free radicals and phospholipids [13]. Flavonoids are reported to possess antiulcer activity, hepatoprotective activity, anti-inflammatory activity, antidiabetic effects, vasorelaxant process, anti-atherosclerotic effects, anti-thrombogenic effects, cardio-protective effects and anti-neoplastic activity. Flavonoids are also known to possess antibacterial properties.

In conclusion, the methanol extract exhibited the strongest cytotoxic activity against HepG2 amongst all the *C. nutans* extracts with a high total flavonoid content. Based on these results, study are ongoing to determine the chemical profiling of flavonoids in methanol extract by using UPLC/QToF-MS. A major content of flavonoids may be utilized as an anticancer pharmaceutical application.

ACKNOWLEDGEMENT

Support for this work was provided by Ministry of Education via a research grant (RDU 140102).

CONFLICTS OF INTERESTS

Declared none

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How to cite this article

- Hazrulrizawati Abd Hamid, Izzah Hayati Yahaya. Cytotoxicity of *Clinacanthus nutans* extracts on human hepatoma (HepG2) cell line. Int J Pharm Pharm Sci 2016;8(10):293-295.

APPENDIX B: AWARD

Universiti Malaysia PAHANG

ITREX

Certificate of Award

Bronze Medal

This Certificate of Award is presented to

Dr Hazrulrizawati Bt Abd Hamid, Izzah Hayati Bt Yahya

For the invention / innovation of

Chewable CliNu, Sulfur Health Supplement

CREATION, INNOVATION, TECHNOLOGY & RESEARCH EXPOSITION 2016
7th – 8th March 2016, Universiti Malaysia Pahang

PROFESSOR DR. MASHITAH MOHD. YUSOFF
DEPUTY VICE CHANCELLOR (RESEARCH & INNOVATION)
UNIVERSITI MALAYSIA PAHANG

APPENDIX C: POSTGRADUATE SUPERVISION

Universiti Malaysia PAHANG

Universiti Malaysia Pahang
Lebuhraya Tun Razak,26300 Gambang
Kuantan, Pahang Darul Makmur
Tel: +609-549 2050/2034 | Faks/Fax: +609-549 2662
e-Mel/e-Mail : ips@ump.edu.my

Institut Pengajian Siswazah
Institute of Postgraduate Studies

UMP.20/13/13.10/1/15/ 12 SEP 2014

Izzah Hayati Bt Yahya
Batu 15 , Lorong Kampung Ayer Merbau,
77200 Bemban
Melaka
Malaysia

OFFER FOR GRADUATE ADMISSION

We are pleased to offer you a place in our graduate programme as stated below.

Faculty	: Faculty of Industrial Sciences & Technology
Programme	: Master of Science (Industrial Chemistry)
Programme Mode	: Research
Topic of Research	: Isolation and Characterization of Apoptosis- Inducing Compounds From Clinacanthus Nutans
Candidature Type / Period	: Full Time / Min : 2 Sem Max : 6 Sem
Pre-requisite for Registration	: You are required to sit for UMP English Proficiency Test (EPT) within the first six months of your studies.
Main Supervisor (MS)	: Dr. Hazrulrizawati Binti Abd Hamid
Validity of Offer	: One (1) Year

We take this opportunity to congratulate you on this offer. Please confirm your acceptance of this offer letter by completing the form through online. Registration is open all year round. Please do not hesitate to contact us at 09-5492599 / 2034 for futher assistance (Please see attachment for futher details of the offer).

Yours sincerely,

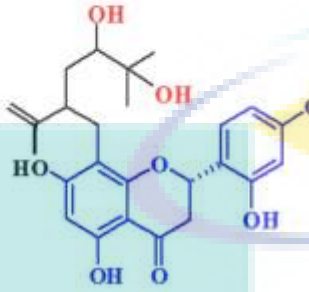
RAJA ALLEN JORDAN IZZUDDIN SHAH BIN RAJA BAHARUDIN
Assistant Registrar

cc Dean
Faculty of Industrial Sciences & Technology
(MS)Dr. Hazrulrizawati Binti Abd Hamid, FSTI
Bursary

14090554942

APPENDIX D: CONFERENCE

PSE
Phytochemical Society of Europe



Phytochemical Society of Europe meeting

2nd International Symposium on Phytochemicals in Medicine and Food

2-ISPMF

April 7-10, 2017, Fuzhou, China

Dr. Hazrulrizawati Abd Hamid
Faculty of Industrial Sciences & Technology
Universiti Malaysia Pahang
Malaysia.
E-mail: hazrulrizawati@ump.edu.my

November 21, 2016

Dear Dr. Hamid,

It is our great pleasure to announce the **2nd International Symposium on Phytochemicals in Medicine and Food** (2-ISPMF) to be held in Fuzhou, China from April 7-10, 2017. 2-ISPMF is co-organized by the Phytochemical Society of Europe (PSE), Phytochemical Society of Asia (PSA), and International Society for Chinese Medicine (ISCM). It dedicates to creating a stage for exchanging the latest research results in phytochemicals for food and human health.
A number of eminent scientists have agreed to participate in the event. Please find more information in http://www.2017ispmf.com/index.html .

Due to your eminent expertise on bioactive functional molecules from medicinal plants, we would like to invite you to participate and deliver an oral presentation "Antioxidant and antiproliferative properties of methanol extract of *Clinacanthus nutans* and identification of chemicals profile by UPLC-QTOF-MS/MS" (20 minutes duration) in the Symposium. Your abstract has been accepted.

We are looking forward to seeing you in Fuzhou and hope you will enjoy a nice travel to China.

Sincerely,

Jianbo Xiao, Executive Chairman
University of Macau, China

Antioxidant and antiproliferative properties of methanol extract of *Clinacanthus nutans* and identification of chemicals profile by UPLC-QTOF/MS

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Abstract

Introduction

The leaves extract of *C. nutans* have been used extensively as primary sources of complementary and alternative healthcare or as economical in-housing regimens for cancer patients [1]. Patients have claimed that they have recovered from cancer illness after consuming C. nutans leaves over a period of time. It has been proved that many antioxidant substances have anticancer or anticarcinogenic properties [2]. A study had pointed to the necessity of chemicals profiling and evaluation of antioxidant and antiproliferative properties of *C. nutans* extract.

Materials and Methods

The whole plant of *C. nutans* were dried and extracted with methanol. The content of the active components in the extracts was determined by ultrahigh performance liquid chromatography-quadrupole time-of-flight/mass spectrometry (UPLC-QTOF/MS). The compounds were tested for antiproliferative property on HT29, HepG2 and MCF-7 cell lines while antioxidant activity was monitored by radical scavenging assay (DPPH) and ferric reducing power (FRAP).

Results

The content of the active components in the extracts were determined by UPLC-QTOF/MS.Six compounds were labelled as confirmed component from methanol extract of *C. nutans*. Peaks with retention times (RT; min) of 4.36, 4.94, 6.63, 7.60, 8.69 and 11.27 were identified as the following: 5,7-dihydroxychromone-7-β-D-glucoside, smiglanin, glabrol, corymboside, viscumneoside II and kushenol U (Figure 1). Based on the component confirmed plot, the major compound found was corymboside. Methanol extract show significant antioxidant activities in DPPH (IC50 = 127.09± 0.042 µg/mL) and total reduction capability (IC50 469.13 ± 0.05 µg/mL) (Table 1) According to the IC_{50} obtained, the methanol extracts showed significant antiproliferative activity on HT29 (IC_{50} 36.19 ± 1.06 mg/mL), HepG2 (IC_{50} 48.37 ± 0.026 mg/mL) and MCF-7(IC_{50} 54.16 ± 0.99 mg/mL) after 72 h of treatment (Table 2).

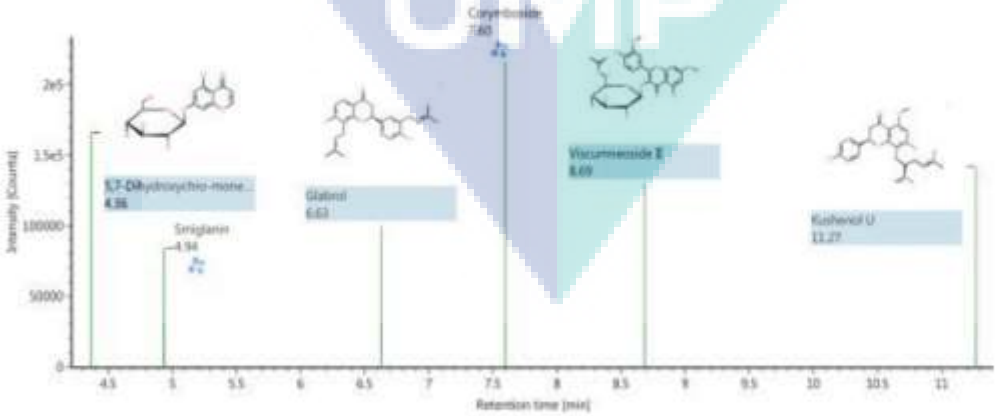

Figure 1 Confirmed component plot of *C. nutans* extract

Table 1 IC_{50} values of DPPH and FRAP assays of *C.nutans*

Extract	IC_{50} values (µg/mL)	
	DPPH	FRAP
Standard	135.02± 0.052	405.15 ± 0.16
Methanol	127.09± 0.042	469.13 ± 0.05

Table 2 IC_{50} values after 24, 48 and 72 hours of incubation of *C. nutans* extracts against HepG2, MCF-7 and HT 29 cancer cell lines

Cell lines	**IC_{50}(µg/mL)**		
	24hr	**48hr**	**72hr**
MCF 7	ND	ND	54.164±0.991
Hep G2	84.080±0.461	94.825±1.511	48.370±0.026
HT 29	72.11±1.036	50.11±0.864	36.19±1.058

Conclusion

These results suggest antioxidant and antiproliferative properties of *C. nutans* extract might due to the presence of chromones, flavones and their glycosides as evidenced from UPLC-QTOF/MS.

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