Recent methods for extraction and identification of *Clinacanthus Nutans*

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Clinacanthus Nutans

Clinacanthus nutans (C. nutans) which commonly called as Sabah snake grass or "belalai gajah" by the local Malaysian is a small shrub which belongs to the family Acanthaceacae. As stated by other researchers, this plant commonly found in South East Asia especially in Thailand, Indonesia as well as Malaysia (Sekar and Rashid, 2016). As revealed by Shim et al., (2013) and Alam et al., (2016), *C. nutans* and *Clinacathus siamensis* usually get confused by the people because of their similar appearance. Figure 1.1 shows the branches of *C. nutans* and *C. siamensis* which are quite similar in terms of appearance. According to Kunsorn et al., (2013), *C. nutans* can be identified by observing the leaves. Leaves for *C. nutans* are shorter and pale green compared to *C. siamensis*.

Figure 1.1: Branch of plants (a) *C. nutans*, (b) C. siamensis (Kunsorn et al., 2013)

Acanthaceae is known as a family of dicotyledonous flowering plant. As stated by Alam et al., (2016), this Acanthaceae family has around 250 genera and 2500 species. Apart from that, Acanthaceae is one of the largest suppliers for medicinal plants that could give traditional medicine to cure certain diseases. This type of plant can grow in an open forest, bushes as well as mangrove areas. The Encyclopaedia Britannica (2018) stated that the Acanthaceae have simple leaves that in an opposite pairs arrangement and it also has an enlarged cell containing crystal of calcium carbonate or commonly known as cystoliths.

Recently, researchers have shown an increased interest of the useful, chemical composition of *C. nutans.* For instance, Barek et al., in 2015 studied the impact of drying method towards unfermented as well as fermented *C. nutans* herbal tea. In summary, this research has concluded that different method causes different changes in composition. Apart from that, Shim et al., (2013) did research about the ointment formulated from *C. nutans* extract. This research focused on the effect of the ointment towards skin diseases.

One of the most current significant discussions for *C. nutans* is the biological properties as well as the phytochemical in the *C. nutans*. Alam (2016) has proved in his research that *C. nutans* consists of biological properties such as antibacterial, antioxidant, antiviral, as well as antidiabetic activities. There is also research that has proved these biological properties in *C. nutans.* For instance, Zulkipli et al., (2017) stated that anti-proliferative, anti-tumorigenic as well as anti-inflammatory also some biological properties that can be found in *C. nutans* and is proven. Therefore, by having these beneficial biological properties, *C. nutans* is believed to cure cancer, relieve pain and itchiness as well as controlling diabetes. Another research that is also essential in this study is the presence of a phytochemical compound in *C. nutans.* Alam (2016) revealed that bioactive compound such as flavonoids, glycoside, and glycoglycerolipids are the example of phytochemical exists in the plants. In addition to other research such as Sekar and Rashid, (2016) and Shim et al., (2013), steroid, tannins, flavonoids, and saponins are other phytochemical that can be found in the extraction of *C. nutans*.

History of *Clinacanthus Nutans*

Clinacanthus nutans is a small shrub that has attracted much attention as it is a very well-known traditional herb and plant that has few vernacular names based on the different region in Asian. In Malay, it is called 'Belalai Gajah' while in China it is called 'E Zui Hua,' 'She Be She Cao' and 'You Dun Cao' (Ying, 2013). Besides, *C. nutans* commercially known by the name of 'Payayor', 'Saled Pangpon Tua Mea' in Thailand (Watson & Preedy, 2008) and even in Javanese it has a unique name different from other region called 'Kitajam'

(Khoo et al., 2018). Nowadays, the *C. nutans* plant has major roles to human because of its unique characteristics that make it able to cooperate well in the pharmacological activity such as the anti-inflammatory, anti-microbial and anti-venom activities. There is some report claimed that it could cure cancers but the proof of its efficiency is not well spread, and the amount of research conducted is countable, yet the evidence is not much.

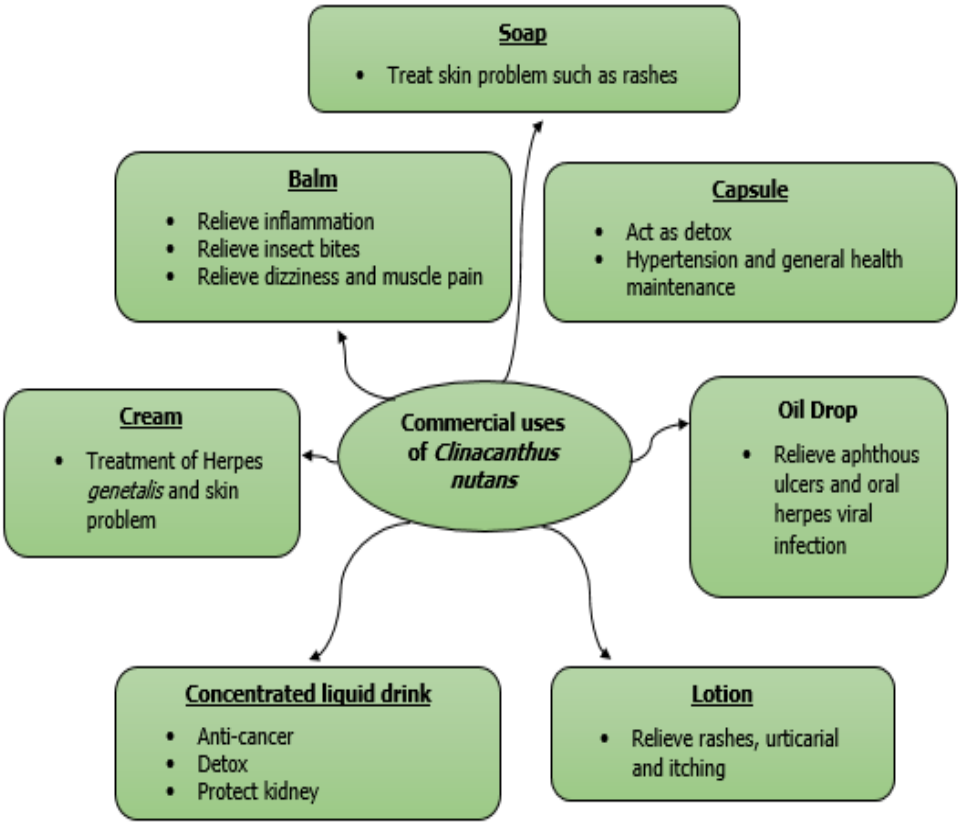
Applications of *Clinacanthus Nutans*

This century, *C. nutans* has been commercialized throughout the country especially southeast Asia. As stated by Sekar and Rashid (2016), *C. nutans* is one of the plants that can be found in Sabah which eventually carried the name Sabah Snake Grass. However, due to its profound effect, it has been introduced and cultivated in all the state of Malaysia. Several studies have revealed that *C. nutans* has been commercialized due to its beneficial value such as curing cancer, as well as antibacterial properties. Zulkipli et al. (2017) reported that a total of 10 products had been commercialized in the market made from *C. nutans* extract. The author has shown the product as well as their benefit to the health of human being. Other studies conducted by Shim et al. (2013) stated that the product such as soap, herbal teas, capsule as well as plant extract could be found throughout the market.

In other countries, as summarized by Shim et al. (2013) *C. nutans* has been approved by Ministry of Thailand as one of the medicinal plants to treat skin rashes. Thus, the formulation of capsules as well as the cream has been made recently. Moreover, as reported by Charuwichitratana (1996), a cream made from *C. nutans* has successfully passed the clinical test to relieve skin inflammation that usually caused by *Staphylococcus aureus*. This is because, *C. nutans* is known as a plant that contains antibacterial properties (Sekar and Rashid, 2016). Besides, Alam et al. (2016) found that this plant is economically essential for pharmacology as well as for herbal products. Besides, cream formulated from dried leaves of *C. nutans* could relieve pain and cure herpes infection.

Next, based on the research of Barek et al. (2015) there are two types of herbal teas made from *C. nutans* known as unfermented and fermented *C. nutans* herbal teas. As revealed from the research, unfermented herbal tea contains Total Phenolic Compound compared to fermented tea. Although this product has not been commercialized yet, this research can be a reference to formulate herbal tea extracted from *C. nutans.* In summary of this research, a newly formulated ointment extracted from *C. nutans*. In general, skin-related disease such as rashes, eczema, and psoriasis are caused by bacteria. As *C. nutans* is known for its antibacterial properties, this ointment has been developed and tested for its effect. This research has shown a positive result whereby it can cure skin rashes. Therefore,

this ointment can be used for commercial production (Sekar and Rashid, 2016). The commercial uses of *C. nutans* are simplified below.



Phytochemical of *Clinacanthus nutans*

Bio-reactive compounds

Phytochemicals are a chemical compound that is non-nutrient based from the plant which did not provide any essential nutrient to the human body, but it is beneficial where it gives a good effect in the chronic diseases that occurred (Huang et al., 2015). In *C. nutans* it contains few bio-reactive compounds can be found in different type such as Stigmasterol that has a similar chemical structure to cholesterol that exhibits such anti-inflammatory and anti-cancer like stated in the international journal of pharmaceutical science and research (Kaur et al., 2017). Stigmasterol is a sterol type of phytosterol or also known as a plant sterol that also includes the triterpenoids because it has its biological activities whereas the stigmasterol able to lower the blood cholesterol level and at the same time, it has a contribution in the oxidation and serum lipid where the anticancer properties can be exhibit by the presence of stigmasterol (Peter et al., 2009). B-Sitosterol that has a similar chemical structure as stigmasterol but differ in the saturated side chain with only one double bond in the cyclohexane ring (Suttiarporn et al., 2015).

Charuwichitratana et al. (1996), revealed that there is another bioactive compound present in the *C. nutans plant* named lupeol compound. It claimed that lupeol is a kind of dietary triterpener that has a major role in cancer treatment. It acts as a bio-active compound that inhibits proliferation and also induce apoptosis (Liu et al., 2015). Aslam et al. in 2015 stated in a report that the *C. nutans* contained the phenolics, benzenoids that could be obtained by the GS-MC analysis, besides, glycoglycerolipids and glycostlglyceride also obtained along with the fatty acid compound which is the oleic acid and the sulfur-containing. Apart from that, a mixture of nine cerebrosides and monoacylmonogalatos that obtained from the method of extraction of methanol also presented as a bio-reactive compound (Tuntiwachwutikal et al., 2004). Cerebrosides on the other hand defined as a lipid or also known as a glycoapoptosis same like the lupeol compound that involves in the modification of the apoptosis which is the link to cancer issues. Cerebrosides has a benefits of reducing the low-density lipoprotein (LDL) make up almost in body's cholesterol that has a property to harm human health such as heart diseases and stroke and did the opposite thing where it elevates high-density lipoprotein (HDL) where it should be kept higher (Vesper et al., 1999).

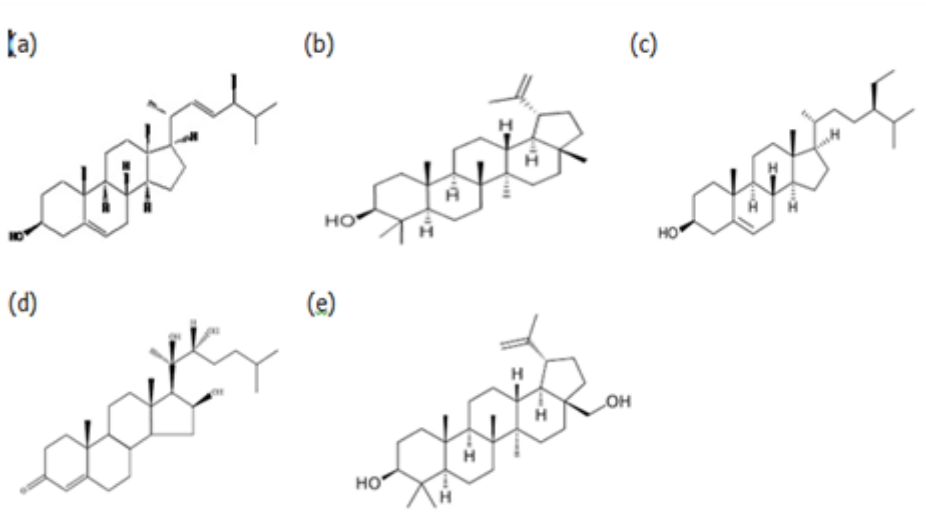


Figure 2.1: Chemical structure of bio-reactive compounds in *C. nutans* (a) Stigmasterol, (b) Lupeol, (c) Sitosterol, (d) Mycricyl alcohol, (e) Betulin (Charuwichitratana, 1996; Dampawan, 1976; Lin et al., 1983).

Recent research about this bio-reactive compound reveals that more compounds were found in ethyl acetate root extract compared to methanol root extract. The differences in the extraction method, detection method, and plant method may impact the extractability and identification of certain compounds (Teoh et al., 2017). The mixture of the n-BuOH, or also known as n-butanol or butyl alcohol with water-soluble portions of methanol isolates six known *C-glycosyl flavones* such as Vitexin, Isovitexin, Schaftoside, Orientin, and Isoorientin. Other than that, there are 5 sulphur containing glucosides known as clinacoside A, clinacoside B, clinacoside C, cycloclinacoside A1 and cycloclinacoside A2 obtained by the same method. Meanwhile, in the ethanol extract of the aerial part, four new sulfur is obtained namely Clinamades A, Clinamades B, Clinamades C and 2-Cis-entadamine A also three sulphur containing compound is isolated known as entadamide A, entadamine C and the last sulphur containing compound Trans-3-methylsulfinyl (Teshima et al., 1998).

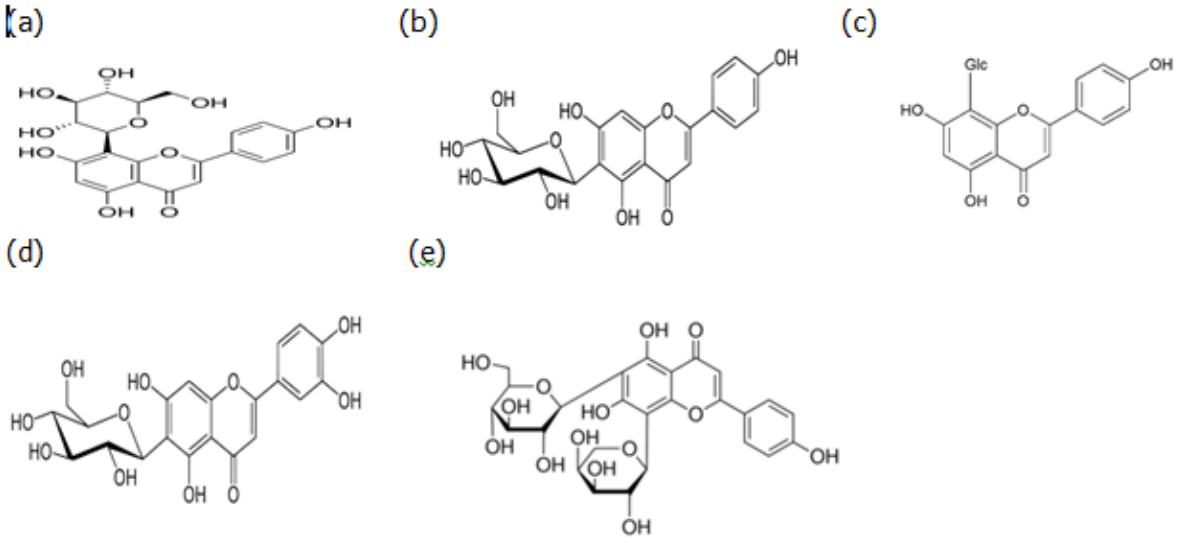


Figure 2.2: Chemical structure of *C-glycosyl flavones* (a) Vitexin, (b) Isovitexin, (c) Orientin, (d) Isoorientin, (e) Schaftoside (Charuwichitratana, 1996; Dampawan, 1976; Lin et al., 1983).

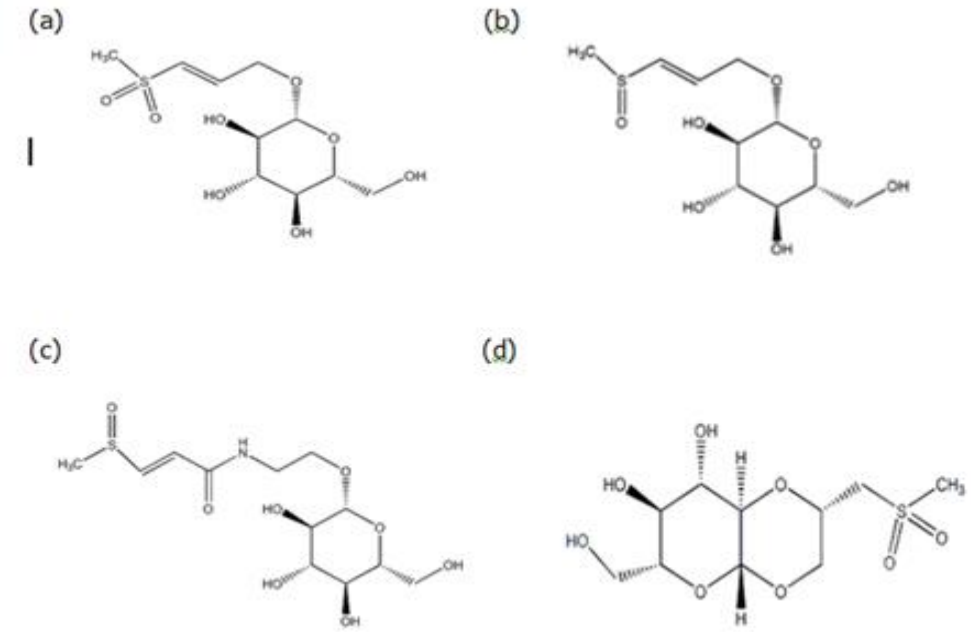


Figure 2.3: Sulphur containing compounds (a) clinacosides A, (b) cycloclinacosides b, (c) cinacosides C, (d) cycloclinacosides A1 and cycloclinacosides A2 (Teshima et al, 1998)

Pharmacological of *Clinacanthus nutans*

Anti-inflammatory

The anti-inflammatory agent is defined as a drug or substance that can reduce inflammation such as redness, swelling, and pain in the body. It blocks certain substances in the body that causes inflammation. *C. nutans* is already used by the people in the South East Asia country such as Thailand as an anti-inflammatory agent for the treatment of insect bites and allergic responses. Several studies have already been made that the extracts from *C. nutans* leaves can be used to reduce symptoms of inflammation. Sriwanthana et al. (1996) found out that at low doses, Clinacanthus extract give effect to the peripheral blood mononuclear cells proliferation by increasing its activity which can be a potential mitogenic property. Alternatively, the effect of inflammatory can be reduced if the doses of the extracts are also higher as an inflammatory cytokine, interleukin-4 is only effective at higher doses. Furthermore, another finding by Wanikiat et al. (2008) also find a breakthrough regarding the anti-inflammatory effect of *C. nutans* extract through their research on the reduction of oedema in the ears and paws of two rat models that indicate the inflammation activity.

Antioxidant

Antioxidants are substances that neutralize potentially damaging oxidizing agents or free radicals, which could be the factor to cause chronic health problems in diseases such as cancer, cardiac disease, and aging-related disorders. There are considerable numbers of analyses that have been done relating to the antioxidant effect of *C. nutans*. The method of extraction can affect the antioxidant value in *C. nutans* which shown by the findings of Ghasemzadeh et al. (2014) that which the bud was extracted, underwent freeze-drying, and methanol extraction shows the high antioxidant property. Other than this, another finding shows that *C. nutans* extracts can reduce oxidative free radical production by phorbol 12-myristate 13-acetate (PMA)-stimulated rat macrophages significantly (Pannangpetch et al., 2007) Furthermore, the extract also showed a substantial inhibitory effect on haemolysis in a 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH)-induced cell lysis model. AAPH will cause the breakdown of red blood cells through oxidation of lipids and proteins in the blood cell membranes. However, an antioxidant activity more likely to show in a polar and semi-polar solvent-extracted *C. nutans* than nonpolar extracts (Khoo et al., 2018).

Anti-Microbial

Anti-fungal

Fungi is defined as the kingdom which usually consists of multicellular eukaryotic organisms that are heterotrophs. It is also may be responsible for being the cause of diseases towards animals and plants. An example of fungi is *Candida* species which can cause candidiasis that is usually occurring in cancer patients after they had their surgery or therapy. In South East Asia, Thailand specifically, herbs such as *C. nutans* widely used as a traditional pharmaceutical that can overcome the fungal activity. However, there a few types of research that can support the claims that were made by the people. On the other hand, the research that is made show some positive result.

In the research that was made by Choonharuangdej et al. (2014) regarding the anti-Candida effect of Thai herbs shows that *C. nutans* did show inhibitory activity against *Candida albicans* which is a type of fungi by agar disk diffusion. However, when the *C. nutans* aqueous were supplemented in a COE-COMFORT tissue container, which to indicate a real-life situation, it does not show any anti-Candida activity. This was later supported by Khoo et al. (2018), that stated that *C. nutans* has a few types of research regarding its antifungal activity and in the two researchers that are made by Cheeptam and Towers in 2002 only shows negative result as *C. nutans* did not show antifungal activity towards *C. albicans* or *A. fumigatos*. The conditions that obtained these results are 95% ethanol leaf extract at 5 mg/mL but the use of ethyl acetate extract at a minimal concentration of 1.39 mg/mL exhibit a fraction of antifungal effect on *C. albicans* (Arullappan et al., 2014).

Anti-bacterial

Anti-bacterial is defined as anything that either could destroy bacteria or suppresses their growth or the ability to reproduce. Such examples of anti-bacterial are heat, chemicals such as chlorine and antibiotic drug. There are several studies regarding the anti-bacterial effect of *C. nutans*. The studies were tested on microbial strains which have mixed findings (Arullapan et al., 2014). Some reported that *C. nutans* extract inhibits bacterial growth and survival while some finding says otherwise. The most effective extract of the plant was prepared by oven-drying and undergoing acetate fractionation. This method of extraction prevented the growth of B. cereus, E. coli and S. enterica Typhimurium (Khoo et al., 2018).

Anti-cancer

Anti-cancer is described as natural methods of health care which contribute to preventing the development of cancer. Anti-cancer is a complement to conventional approaches such as surgery, radiotherapy or chemotherapy. There is a considerable number of studies and analyses that show the anti-cancer effect of *C. nutans*. However, there are some mixed results obtained through these studies. Huang et al. (2015) reported that Clinacanthus ethanol extract could reduce tumor growth significantly compared to the control treatment of fluorouracil which is an established chemotherapeutic drug in HepA hepatoma model mouse. However, in the findings of Teoh et al. (2017), *C. nutans* root extracts are selective towards cancer cells, and it will not affect the proliferation of the normal cell line. Furthermore, different extraction method also shows different results which ethyl acetate root extract promoted mitochondrial-dependent apoptosis in MCF-7 cells, but this was not shown in cells that are treated with methanol root extract. In other findings also shows the same result regarding of different extraction method in which aqueous extract from leaves of *C. nutans* possessed cytotoxic effects against human cervical cell lines, HeLa compared to methanol extract with no cytotoxic effects towards a non-malignant cell line, Vero (Zakaria et al., 2017).

As *C. nutans* is quite well-known among the traditional medicine practitioners for its various beneficial properties, therefore there have been some studies conducted on the effectiveness of *C. nutans* with regards to the various claims made. One of the claims is on the ability of *C. nutans* in treating and curing cancers in regards to the anti-cancer and anti-proliferative properties of *C. nutans.* For this purpose, *C. nutans* has been extracted from different parts of the plants such as leaves, stems (Fong, 2015) and also the roots (Teoh et al., 2017). It was also extracted in different types of solvents, and it was found out by Kamarudin et al. (2017) that semi-polar extracts of *C. nutans* show better cytotoxicity than polar extracts of *C. nutans. C. nutans* extracts have been treated with various cancer cell lines such as cervical cancer cell (HeLa), lymphoma cell line (Raji), erythroleukemia cancer cell (K-562) (Yong et al., 2013), breast cancer cell (MCF-7) (Teoh et al., 2017) and also D24 melanoma cell (Fong et al., 2016). However, only the effects of treatment towards cervical cancer, breast cancer, and melanoma skin cancer will be further discussed in this section.

Basically, the effectiveness of *C. nutans* extracts in treating cancer cells is associated with the induction of apoptosis, which in general apoptosis is a cell death process which occurs under certain circumstances such as 1) the result of aging and body development, 2) the result of homeostatic mechanism, 3) or the act as defensive mechanism (Elmore, 2007). Other than that, it is also associated with cytotoxicity, which is the ability to inhibit the

multiplication or growth of cancer cell (Zakaria et al., 2017) which demonstrated by anti-proliferative activity. However, the results of the treatments are much dependent on the concentration of *C. nutans* extracts and the treatment duration (Fong et al., 2016).

Cervical Cancer

In the studies conducted by Zakaria et al. (2017), *C. nutans* was extracted from the leave, while using aqueous and methanol solvents. The finding of the study was the presence of terpenoids and flavonoids in aqueous extracts, while alkaloid was only found in methanol extracts. These phytochemicals have a great contribution towards the anti-cancer properties of *C. nutans* which are apoptotic effect and cytotoxic effect. However, only aqueous extract showed toxicity effect towards HeLa (cervical cancer cell), which is suggested that the presence of alkaloid in methanol leave extracts is somehow arresting the toxicity effect.

HeLa cell has been treated with both of leaves extracts of *C. nutans*, while in other reports by Teoh et al. (2017), HeLa cell was treated with methanol and ethyl acetate extracts from the roots of *C. nutans.* Both studies reported that the extracts of *C. nutans* can promote cell cancer death by inducing apoptosis, other than exhibiting the cytotoxic effect. However, the result of the study shown that HeLa cell is found to be less susceptible towards the methanol and ethyl acetate roots extracts, as compared to breast cancer cells.

After being treated by aqueous extracts from *C. nutans* leaves, HeLa cells have been observed with apoptosis morphology characteristic which is the presence of chromatin condensation, nuclear fragmentation and apoptotic bodies (Zakaria et al., 2017). Other study made by Yong et al. (2013) also shown that the aqueous extracts of *C. nutans* leaves give significant cell proliferation towards HeLa cell.

The positive outcome from the treatment of aqueous extracts is believed to be contributed by the presence of terpenoids and flavonoid compound. According to Zakaria et al. (2017), there are 10 beneficial health effects of terpenoid. However, the emphasized property is that terpenoid possess the most anti-cancer activity as claimed by the FDA. Meanwhile, flavonoid has been found from the study that it also contributes to treating cervical cancer as it acts as an anti-proliferation agent, other than associated with apoptotic effect induction.

The leaves extract of *C. nutans* might be more efficient in treating cervical cancer, rather than roots extracts of *C. nutans* as different plant parts possess different types of compound that are associated the most with anti-cancer properties (Teoh et al., 2016). On

the other hand, aqueous extract of *C. nutans* leaves are found to have more significant anti-proliferation and apoptotic effects as compared to methanol extracts of *C. nutans* leaves.

Breast Cancer

Breast cancer cell which is MCF-7 has been treated with methanol and ethyl acetate root extracts, and the study conducted shown that both extracts significantly inhibit the growth of MCF-7. The apoptotic effect is demonstrated by the changes in morphological characteristics after the treatment. However, it was found that ethyl acetate extract is more effective in inhibiting the proliferation of MCF-7, in which peripheral nuclear membrane condensation, cell shrinkage, loss of cell contact and the condensation of chromatin is more obvious (Teoh et al., 2017) after the treatment of ethyl acetate extracts rather than methanol extracts.

According to Teoh et al. (2017)**,** apoptosis pathway is divided into two categories which are an extrinsic and intrinsic pathway, in which BAX controls intrinsic pathway (promotes apoptosis) and BCL-2 proteins (inhibit apoptosis). Lupeol which was found to be the greatest amount of compound contained in both extracts is suggested to be the contributing factor which inhibits the anti-apoptotic (BCL-2). Thus the inhibition of cytochrome c release from mitochondria is arrested. On the other hand, Lupeol also promotes the expression of pro-apoptotic (BAX) which helps the mitochondria to release cytochrome c. The author added that even-though both extracts contain Lupeol which associated in MCF-7 growth inhibition, however, methanol root extracts is found to be less effective than ethyl acetate extract, as apoptosis induction in methanol extracts does not involve Lupeol very much.

Melanoma Skin Cancer

In the study conducted by Fong et al. (2016) on D24 melanoma cancer cell line, methanol extracts of *C. nutans* leaves were used in the treatment. Melanoma cancer is the least known among skin cancers; however, it is fatal and can cause death to the patients (Fong, 2015). Based on the study, D24 cell was treated with 1 mg/mL, and 2 mg/mL and the results were observed in 24 hours and 72 hours later.

Based on the morphological observation on the treated D24 cell, Fong et al. (2016) reported that the methanol extract was able to induce apoptosis activity in which such changes as shrinkage in a cancer cell, condensation of chromatin and segmented nucleus. The author also suggested that the cytotoxic activity from the leaf extracts of *C. nutans* is associated with flavonoid compound which arrests cell proliferation and promotes cell death.

Anti-diabetic

Type-2 diabetes is generally characterized by a progressive decline of insulin action, and it is the most prevalent type of diabetes. Anti-diabetic agents are substances that can help a person with diabetes to control their level of glucose in the blood. Such examples include insulin and oral hypoglycemic agents. It is believed that *C. nutans* can be used as a treatment of diabetes. The initials findings show the poor result in which the methanol extract of leaf and stem demonstrated a weak α-glucosidase inhibitory activity even at high concentrations (Lee et al., 2014). However, after further research regarding its anti-diabetic property that Clinacanthus extract was only effective against α-glucosidase when used at a high concentration that using different treatment from the initial finding. The treatment used is 70% ethanol extract of oven-dried Clinacanthus leaves with sonication showed higher inhibition of α-glucosidase activity (Wong et al., 2014).

Analgesic activity

Analgesic is an agent that diminished sensation to pain without loss of consciousness. It is also a drug that can be used to relieve pain and produce analgesia. Examples of analgesics are aspirin and acetaminophen that are used for the treatment of pain caused by cancer. *C. nutans* is believed to possess analgesic property. This believed was then supported by findings in some analysis. The findings suggested that *C. nutans* n-butanol extract at 90mg/kg as well with methanol extract at 279.3 mg/kg shows analgesic properties in comparison with the treatment of phenylbutazone at 100 mg/kg (Satatyavivad et al., 1996). Other findings with different types of assay suggested that the methanol extract with a half-maximal effective concentration at 227.7 mg/kg can relieve pain in the late phase. Furthermore, there are also studies that comparing between methanol, ethanol and n-butanol extract of *C. nutans* in which methanol extracts are more effective in alleviating the pain response when compared to ethanol and n-butanol extract as there are no analgesic effects shown (Rahim et al., 2016).

Ethnomedicinal of *Clinacanthus nutans*

Traditional uses

For a long time ago, drugs or medicine usually are inaccessible and very high in cost. This situation has made people make their traditional remedies that led to the utilization of plant as a source of medicine. Khoo et al. (2018) stated that large development in investigating the herbal plants as alternative therapeutic agents for a different type of diseases is notable,

starting from the late 1980s and continuing in recent years. Alam et al. (2016) stated that *C. nutans* had been traditionally used for a long time ago in a different region of Asia due to their different pharmacological effect. In Malaysia for an instant, they consume *C. nutans* as an herbal tea by boiled its fresh leaves with water which it is believed can give a good effect for dialysis and treating kidney dysfunction and help to detoxify the body. Meanwhile, Faris et al. (2016) stated that after the disease is diagnosed, *C. nutans* is commonly used as an herb and as an alternative to the current medicine. It helps to decrease blood pressure, lowering high level of blood cholesterol, glucose and even uric acid. Although it was practiced a long time ago, only in 2011 this plant began gaining popularity in Malaysia, and various types of *C. nutans* related studies have been conducted by Malaysian scientists (Khoo et al., 2018).

In contrast with Malaysia, the government of Thailand has recommended using *C. nutans* as one of the herbs in hospitals since 1987, and it was included in their Primary Health Care Programme. Thailand used it for external treatment such as skin rashes, snake and insect bites, herpes simplex virus (HSV) and also varicella zoster virus (ZVZ) infections by using the alcoholic extraction from the *C. nutans* leaves. It is also prominent in Thailand as anti-snake venom amongst the traditional healer (Alam et al., 2016). Meanwhile in China, as mentioned by Faris et al., (2016), they traditionally treat several of the inflammatory condition by using recipes that involved a different part of *C. nutans* either individually or combined with other herbs. Several medical conditions have been treated by utilizing the combination of *C. nutans* with another type of herbs. In China, they also use the whole plant in various inflammatory conditions like a hematoma, contusion, strain, and sprain of injuries and rheumatism (Alam et al., 2016).

Anti-Venom

It has been ages in Thailand and North-Western Malaysia, *C. nutans* has been traditionally used as medicine for envenomation snakes and venomous insects such as scorpion and bees. Stated by Arullapan et al., (2014), *C. nutans* has been used as anti-venom for snake and also its bites because it contains anti-cell lysis properties. There are two ways on how to use the fresh leaves of *C. nutans* by internal consumption where it is 20-25 *C. nutans* leaves is chewed, and the other way is by external uses, *C. nutans* leave left and pounded in the snake bites area. Since *C. nutans* has been claimed that it has anti-venom properties, few authors such as Khoo et al., (2018) conducted a study about this anti-venom property. Only one out of four of the *C. nutans* has exhibited anti-venom properties; thus it still ambiguous in defining the remedy for snake and insect venom with suitable extract. Meanwhile, Farsi et

al. (2016) did an experiment and obtained that by using water extract of *C. nutans* leaves, it helps against scorpion venom such which is cobra venom used the extraction of *C. nutans* show no protective effect as the lethal activity of the cobra venom cannot be stopped. In summary, the anti-venom properties of *C. nutans* still undetermined as it did not show consistency in stop lethal activity of the venom and could not exhibit anti-venom effects.

Anti-Viral (Anti HSV)

Herpes simplex virus (HSV) is known as a virus that causes genital herpes which is a sexually transmitted disease (Ruhaiyem et al., 2015). According to Stannard et al., (1987), HSV is a double-stranded DNA virus and consist of two classifications of HSV-1 AND HSV-2. As for HSV-1, even though it can cause genital infection as well, but it is usually involved with skin above the waist (Stannard et al., 1987). An individual with HSV-1 infection may have cold sores, encephalitis, corneal damage, blindness and herpetic whitlow (Yeo et al., 2018). Meantime, for HSV-2, it is usually related to the below the waist skin and genital infection in sexually active individuals.

Traditionally, *C. nutans* has been used to treat HSV infections. For an instant, in Thailand, as mentioned by Ruhaiyem et al., (2015) in studies that *C. nutans* were used a long time ago to treat this HSV in that country. The fresh leaves of *C. nutans* have reported by clinical trial effective to treat herpes simplex virus. A further clinical trial has reported that it is effective to treat the HSV-2 infections by using topical preparation containing *C. nutans* extracts. Tuntiwachwuttikul et al., (2004) stated in his study that fresh leaves of *C. nutans* possessed anti-HSV-1 activity when it is extracted with Ethyl Alcohol (EtOH) after going through certain processes. On another hand, Yeo et al., (2018) have also stated the same finding in his study of *C. nutans* which the plant's leaves extract has given anti-HSV properties. He suggested that the intracellular activity of HSV could be affected by the extract of *C. nutans* extract. There are some of the anti-HSV agents that have been recognized from the bioactive constituent of the *C. nutans* such as polyphenolics, glycosides, and terpenes. Based on this finding, it has shown the potential of *C. nutans* as an antiviral agent such as anti-HSV activities. Another study has shown that the topical formulation of *C. nutans* extracts based been stated effective against the infection of HSV- 1 development and its progression (Lipipun et al., 2011). Based on several studies, it can be said that *C. nutans* gives positive effect against HSV infections, for HSV-1 and HSV-2.

Extraction Method

Sonication

Generally, the sonication method is usually combined with, for instance, air drying and oven drying especially freeze-drying. Before undergoing sonication, the leaves of *C. nutans* is the first oven dried at 40°C. Besides, it is important to produce a fine powder of leaves by grinding them. Next, ethanol is added into the powdered raw materials to form ethanolic extract (Chelyn et al., 2014).

After this process, the extract is then sonicated for around 30 minutes and is filtered using Whatman no. 1 filter paper. This is crucial as different methods of extraction and drying might affect the total phenolic content (TPC) in *C. nutans*. Leaf extracts contained more TPC than stem extracts. While sonication and air drying is the best combination of techniques to extract *C. nutans* with the highest phenolic content compared to soak and other drying methods like oven and freezing. In conclude, sonication is a better method compared to soaking to extract *C. nutans* supported with better anti-oxidation properties, α-glucosidase inhibitory properties and higher phytochemical content (Khoo et al*.,* 2015).

Microwave Assisted Extraction (MAE)

Based on Mustapa et al., (2015), the solvent used in MAE method is a mixture of ethanol and water conditioned with a maximum power of microwave oven of 300 W and frequency of 2.45 GHz. The real temperature profile of the process is measured by the optical fiber probe associated with the microwave.

By using MAE, the heat produced by microwave effect will heat up the moisture inside the plant cell. Thus, resulting in evaporation as well as generation of tremendous pressure on cell wall of the plant due to swelling and since a huge amount of pressure is being applied to the cell wall from the inside, the plant cell is stretched and ruptured causing the active constituent of the plant is leached out from cell to the solvent (Mandal et al., 2007). Therefore, this method improves the yield of phytoconstituents.

The solvent used in the MAE method is a mixture of ethanol and water conditioned with a maximum power of microwave oven of 300 W and a frequency of 2.45 GHz. At the same time, MAE is simpler, more efficient and consume less solvent to carry out the extraction process of phyto-compound from medicinal plants (Mustapa et al., 2015). The real temperature profile of the process is measured by the optical fiber probe associated with the microwave. The samples are soaked in the solvent after continuously stirred magnetically for 3 minutes. Magnetic stirring enhances the diffusion of solvent into the

sample matrix and promotes the mass transfer of active compounds into the solvent. Formation of hot spots within the sample can be avoided by constantly stirring the ethanol-sample during microwave irradiation, meanwhile homogenizing the temperature of the mixture during the whole process. Samples are cooled down rapidly to 40°C after irradiation and ethanol are added into the sample to further carry out the extraction in warm water (40°C) for a period.

Soxhlet Method

In general, Soxhlet extractor consist of a thimble in which the sample is placed inside it, a bottom flask which the solvent is heated inside it, a condenser which the evaporated solvent will drip back to the reservoir, and a siphon which pours back the solvent when it reaches the level in the reservoir, to allow the cycle to restart again. In terms of the sample used, the plant sample to be extracted can be in fresh condition or dry condition. However, the sample must be in powdered form, in order to provide a greater surface area (Redfern et al., 2014).

The extraction of *C. nutans* leaves conducted by Roslan et al. (2018) used was in dried form, in which the leaves were oven-dried at 50°C for two days before it was extracted sequentially in hexane and chloroform solvent, in which the sample was soaked in hexane and incubated for eight hours at 60°C before the extraction was continued in chloroform solvent for two days. In other study conducted by Zakaria et al. (2017), the dried crushed *C. nutans* leaves were extracted in distilled water to produce aqueous extracts, in which the sample was soaked in 250 mL distilled water for 72 hours at 60°C, while methanol solvent was used to produce methanol extract in which the sample was soaked in 250 mL methanol for 48 hours at 60°C.

Methanol Extract

Methanol is stated as the best solvent as compared to another solvent such as ethanol, water, acetone, and ethyl acetate when used to extract phenolic content from different parts of the plant. Based on P'ng et al. (2013), methanol solvent is able to extract a higher amount of total phenolic content as compared to other solvent mentioned above. Leaves of *C. nutans* are extracted and producing methanol extracts then the determination of secondary metabolites is performed by phytochemical screening (Zakaria et al., 2017).

According to Tiew et al. (2014), a portion of the dried leaves of the *C.nutans* is dissolved with a certain amount of methanol. It is then extracted by using a Soxhlet extractor or system to obtain the crude methanol extract (Zakaria et al., 2017). The author has also stated the methanol extracts is filtered by using vacuum rotary evaporator at 60°C for 48 hours to concentrate it. After that, the dried extract is weighed and kept and freeze in the refrigerator. The same method that has been studied by Parekh et al. in 2007, the methanol extract is also gained by extracting dried plant powder in methanol and the methanol extract then also keep in a rotary shaker for 24 hours. After filtering the extract, it then centrifuged for 15 minutes at 5000g and dried under low pressure. It is also mentioned by Parekh et al. (2007), the yield obtained of methanol extract is 20.93% of the initial dry material which the methanol extract is kept in airtight bottles at 4°C.

Extraction Method	**Solvent**	**Extracts**	**Reference**
Sonication	Ethanol	Ethanolic extract	Chelyn et al. (2014)
	Methanol	80% methanolic leaf extract	Sarega et al. (2016)
	Methanol and water		
MAE	Ethanol and water	Ethanolic extract	Mustapa et al. (2015)
	Polyphenols	Polyphenols extract	Pan et al. (2003)
	86% Ethanol, 50% Ethanol	Aqueous ethanol extract	Khoo et al. (2018)
Soxhlet	Hexane and chloroform	Chloroform crude extract	Roslan et al. (2018
	Distilled water	Aqueous extract	Zakaria et al. (2017)
	Methanol	Methanol extracts	Zakaria et al. (2017
	Chloroform	Chloroform extracts	Khoo et al. (2018)
Methanol extract			

Table 2.1: Different Types of Extracts from Different Extraction Method

Identification Method

Gas Chromatography-Mass Spectrometry (GC-MS)

This method is a combination of gas chromatography (GC) and mass spectrometer (MS), in which GC is able to separate compound based on the volatility, while MS provides structural information which allows the separated compound to be identified (Hussain and Maqbool, 2014). Based on the study conducted by Cheong et al. (2013), GC (Agilent GC 7890A) and MS (Agilent inert MSD 5975C with Triple-Axis Detector) was used to analyse ethyl acetate and methanol extracts of both stems and leaves of *C. nutans* in which bioactive compounds such as Stigmasterol and Campesterol had been identified. Meanwhile, Yong et al. (2013) used GCMS (GC-MS-QP2010 Plus-Shimadzu) to analyze chloroform extracts of *C. nutans* leaves in which chemical constituents such as 1,2-benzenedicarboxylic acid and mono (2-ethylhexyl) ester had been identified. Other than that, Ng et al. (2017) had also analyzed hexane extract of *C. nutans* using GC-MS in which phytoconstituents as stigmasterol, phytol, beta-sitosterol were identified.

High Performances Liquid Chromatography (HPLC).

HPLC-UV/DAD analysis is performed on a Waters Alliance 2695 (Millford, MA, USA) system which is connected to a Waters 2996 photodiode array detector (DAD) (Chelyn et al., 2014). Besides, at 40°C and using a Kinetex PFP column which is 250x 4.6 mm, 5 μm, Phenomenex, USA, the separation of chromatographic is performed.

According to Chelyn et al. (2014), the solvent system or the mobile phase in this analysis is consist of mixtures of water and glacial acetic acid which is the solvent A and acetonitrile which is the solvent B where before delivering into the system, these solvents are degassed. By using 0.45 μm of the membrane filter, the samples for HPLC analysis is filtered. It is also stated that the flow rate for this HPLC is 0.7 mL/min with 10 μL of injection volume and at 330 nm monitored signal.

On the other hand, there is also has another type of system of HPLC according to its purposes. For an example, in other study by Sarega et al. (2015) has stated that HPLC has been used to analyze selected phenolic compound in crude extracts, which in this analysis, another type of HPLC is used which is Agilent G1310A pump that linked with diode array detector which Agilent, Stevens Creek Blvd Santa Clara, USA is performing the analysis. In this analysis, the LUNA C-18 column which 5mm, 250x4.6 mm (Phenomenex, Torrance, CA, USA) is used in performing chromatography separation. While the mobile phase is the same with water acetic acid as solvent A and acetonitrile as a solvent, thus, an identification or in

analyzing, different brand and column of this system of HPLC may be varied according to its purposes.

Identification Technique	Sample	Compound Detected	Reference
GC-MS	Ethyl acetate and methanol extracts of stems and leaves	Stigmasterol, campesterol, vitamin E, gamma-sitosterol, lupeol	Cheong et al. (2013)
		Lupeol, sitosterol, stigmasterol, botulin, oleic acid	Teoh et al. (2016)
	Chloroform extracts of leaves	1,2-benzenedicarboxylic acid, mono (2-ethylhexyl) ester, heptadecane, heneicosane, behehic alcohol	Yong et al. (2013)
	Hexane extract	Stigmasterol, phytol, beta-sitosterol, vanillin, vitamin E, squalene	Ng et al. (2017)
HPLC			

Table 4.2: Various Types of Compound Detected from Different Technique

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