Malaysian Journal of Analytical Sciences (MJAS)
Published by Malaysian Analytical Sciences Society

Clinacanthus nutans LEAVES DIFFERENT SOLVENT EXTRACTS EFFECTS ON PHYTOCHEMICAL SCREENING, ANTIOXIDANT ACTIVITY AND GAS CHROMATOGRAPHY-MASS SPECTROMETRY STUDY

(Kesan Pelbagai Ekstrak Pelarut Bagi Daun *Clinacanthus nutans* ke atas Pemeriksaan Fitokimia, Antioksidan dan Kajian Kromatografi Gas-Spektrometri Jisim)

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Received: 13 April 2023; Accepted: 3 July 2023; Published: 22 August 2023

Abstract

Clinacanthus nutans (CN) is widely grown in Southeast Asia, especially in Malaysia. People belief that consuming the leaves can treat various diseases it has many medicinal properties. The aim of this study is to investigate the potential of different solvent systems of CN leaves as a source of natural antioxidant by screening its phytochemical constituents and presence of major active compounds with potential biological activities. CN leaves were extracted using different solvent followed by its polarity (methanol, hexane, dichloromethane and aqueous residue). Phytochemical's determination involved total flavonoid content (TFC) and phenolics (TPC) assay. Antioxidant activity was measured using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) scavenging assay and ferric reducing antioxidant powder (FRAP). The chemical constituents of all extracts were identified using gas chromatography-mass spectrometry (GCMS). The percentage of extract yield has more on aqueous fraction (82.84%) and the lower very lower amount of methanol extracts (3.63%). The TFC and TPC were a result of higher and lower phytochemicals content, which were aqueous residue and dichloromethane fractions, respectively. The percentage of scavenging ability of DPPH was higher in aqueous residue (80.994%) and followed by crude methanol extracts (51.829%), dichloromethane fraction (32.426%) and hexane fraction (11.038%). Ferric reducing antioxidant (FRAP), to confirm the existence of antioxidant constituents was found to be higher and a lower activity is shown by aqueous residue and dichloromethane fractions. GCMS was identified as bioactive compounds in all different solvent extracts that indicated various medicinal properties. Thus, aqueous residue and crude methanol extracts CN leaves extracts may be explored as new sources of antioxidants in medicinal plant research. However, dichloromethane indicated a variety of bioactive compounds and medicinal properties.

Keyword: *Clinacanthus nutans*, phytochemical, antioxidant, gas chromatography-mass spectrometry, solvent

Abstrak

Clincanthus nutans (CN) banyak ditanam di Asia Tenggara khususnya di Malaysia. Orang ramai percaya bahawa dengan memakan daunnya boleh merawat pelbagai jenis penyakit kerana mempunyai banyak khasiat perubatan. Objektif kajian ini adalah untuk mengkaji potensi pelbagai ekstrak pelarut bagi daun CN yang menjadi sumber antioksidan semulajadi dengan membuat penyaringan konstitiuen fitokimia dan mengesan kehadiran sebatian aktif utama yang berpotensi dalam aktiviti biologi. Pelbagai jenis pelarut digunakan untuk mengekstrak daun CN mengikut tahap polarity yang berbeza (metanol, heksana, diklorometana dan sisa akueus). Penentuan fitokimia melibatkan pengiraan jumlah kandungan flavonoid (TFC) dan ujian fenolik (TPC). Aktiviti antioksidan diukur menggunakan bahan ujian memerangkap 2, 2-difenil-1-pikrilhidrazil (DPPH) dan serbuk antioksidan penurun ferik (FRAP). Juzuk kimia semua ekstrak yang dikenalpasti telah menggunakan kromatografi gas-spektrometri jisim (GCMS). Keputusan menunjukkan peratusan hasil ekstrak mempunyai lebih banyak pada pecahan akueus (82.84%) dan jumlah peratusan ekstrak yang sangat rendah dalam ekstrak metanol (3.63%). Keputusan TFC dan TPC adalah hasil daripada kandungan fitokimia yang lebih tinggi dan lebih rendah, yang masing-masing merupakan sisa akueus dan pecahan diklorometana. Peratusan keupayaan penghapusan DPPH adalah lebih tinggi dalam sisa akueus (80.994%) dan diikuti oleh ekstrak metanol mentah (51.829%), pecahan diklorometana (32.426%) dan pecahan heksana (11.038%). Ujian FRAP untuk mengesahkan kewujudan juzuk antioksidan didapati lebih tinggi dan aktiviti yang lebih rendah ditunjukkan oleh sisa akueus dan pecahan diklorometana. GC-MS digunakan bagi mengenalpasti kehadiran sebatian bioaktif dalam semua ekstrak ini yang mempunyai nilai perubatan. Oleh itu, sisa akueus dan ekstrak methanol mentah daun CN boleh diterokai sebagai sumber antioksidan baru dalam penyelidikan tumbuhan ubatan. Walaubagaimanapun, diklorometana menunjukkan pelbagai sebatian bioaktif dan mempunyai nilai perubatan.

Kata kunci: *Clinacanthus nutans*, fitokimia, antioksidan, kromatografi gas-spektrometri jisim, pelarut

Introduction

Medicinal plant is one of the biodiversity that can be abundantly found in Malaysia. The excellent source of medicinal plants contains medicinal derivatives and phytochemicals that are beneficial for human health [1]. Medicinal plant plays a vital role in the identification of new beneficial medicinal components and their phytochemical constituents such as antioxidant, anticancer, antihypertension, antidiabetics, antihyperglycemics, antiaging and others. The applications of phytochemicals are widely used in agriculture and cosmetic that has minimal side effects as compared to the use of used synthetic products or drugs [2].

In ancient time, traditional people have been using medicinal herb for the prevention and treatment of disease. Currently medicinal plants are developed as plant derived drug. Various communities use the leaves of CN in Malaysia, Thailand and Indonesia for medicinal purposes such as antiviral, anticancer and anti-inflammatory activities and also to treat fever and diabetes. History of traditional medicine and ethnopharmacological reports of CN are partly established and therefore scientific investigations are required to justify its therapeutic potential [3]. CN is from Acanthaceae family and is known as 'Belalai gajah' in Malaysia. This small plant with full green leaves has a lot of potential phytochemicals such as vitexin, clinamide group, clinacoside group and others [4]. CN also reported to have a lot of antioxidant and benefits of phytochemical compounds. Previous study exhibited CN to have potential flavonoid group such as anticancer agents like quercitine, catechin and luteolin [5, 6]. The pharmacological effects of CN serve as antibacterial, antioxidant, antiproliferative, anti-inflammatory and antiherpes [7]. Traditionally CN in Thailand is used for inflammation, rashes, diabetes, hypertension and diuretic [5, 8].

Plant extraction investigation is important in medicinal plant research for therapeutic purpose to avoid damage to the biomolecules in the plants. The extraction method provides active plant metabolites such as alkaloids, glycoside, phenolics, terpenoids and flavonoids that have natural potential of biological activity. Medicinal plants contain antioxidants that prevent oxidative damage caused by free radicals in the human body [9]. Thus, this study aims to investigate the effects of different solvent extraction of CN leaves on phytochemical, antioxidant and potential of chemical compounds by gas chromatography-mass spectrometry.

Materials and Methods

Plant collections and botanical species identification

The plants of CN were collected and harvested from a farm located at Pongsu Seribu (HERBagus), 13200 Kepala Batas, Seberang Perai Utara, Pulau Pinang. The complete specimen, including roots, flowers, leaves and all parts of plant, was collected (Figure 1). These complete samplings were then sent to Unit Herbarium, School of Biology, and Universiti Sains Malaysia (USM) for voucher specimen preparation of the herbarium. The last step involved herbarium and plant identification by an expert taxonomist from USM. The complete herbarium was deposited with reference number of voucher specimen 111536.

Preparations of plant solvent extractions using partition coefficient

Crude methanol extract and fraction (hexane, dichloromethane and aqueous residue) were prepared using fresh CN cleaned leaves. The process of extraction started with blending the leaves into small pieces and soaking them in a mixture of solvent consisting of 80% methanol and distilled water for 24 h at room temperature. Then, it is filtered and continuously soaked for 2 times to ensure exhaustive extraction. The yield in the form of methanol crude extracts was kept at -20 ^{o}C until it was used. The filtrate was concentrated in a vacuum rotary evaporator at 40^{o}C until about 1/10 of the original volume was left. Then, the leftover filtrate was freeze-dried to obtain the 80% methanol crude extract. The crude was used for successive solvent and partitioning extractions with different solvents of increasing polarity, starting with hexane, dichloromethane and aqueous residue by liquid-liquid extractions. The extracts of partitioning were concentrated to dryness by using a rotary evaporator. The dried powdered extracts were then stored in screw-capped glass bottles and kept in a refrigerator at 4 ^{o}C until further use.

Phytochemical analysis: Total flavonoid content

Total Flavonoid Content (TFC) was determined using aluminium chloride method with some modifications following the method of Kim et al. [10]. The extracts, standard and aluminium chloride ($AlCl_3$) solution were prepared by diluting with distilled water. Then the mixture in the wells was incubated for 10 min in the dark at room temperature. The absorbance was measured at 415nm using microplate reader. All samples were assays in triplicate. The quercitine was used as positive control and standard. The content of total flavonoid compounds was calculated as mean ± SD (n=3) from standard curve quercitine and expressed as milligrams of quercitine (QUE) in 1g of the extract and dried powder.

Total phenolic content

Total phenolic content (TPC) or Folin Ciocalteu assay were determined with modifications of colorimetric method from Sahu and Saxena [11]. Sodium carbonate ($NaHCO_3$) (7.5%) and Folin Ciocalteu reagent (2.5%) were diluted in deionized water. The extracts and gallic acid then were mixed with Folin Ciocalteu and incubated for 5 min. Then sodium carbonate was added into mixture of extracts and Folin Ciocalteu in the well plates. The mixture was incubated about 1h in the dark and measured at 765 nm using microplate reader. Gallic acid was used as standard. The total phenolic content was expressed as mean ± SD (n=3) from standard curve gallic acid and as milligrams gallic acid equivalent (GAE) in 1g of the extract and dried powder.

Antioxidant assay: 2, 2-diphenyl-1-picrylhydrazyl

Free radical scavenging effect of the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) was used to evaluate the antioxidants activity of different solvents extracts of CN leaves as described in the method of Sigma protocol (1898-66-4) (D9132) ($C_{18}H_{12}N_5O_6$) (Mw: 394.33). Trolox was used as a reference compound. In brief, each extract concentration (50 µL) was mixed with 200 µL of DPPH solution (0.2 mM DPPH in 100 mL of methanol). The mixture was incubated in the dark for 30 min at 30 ^{o}C. The absorbance was measured at 517 nm against a blank methanol without the DPPH solution using microplate reader (Biotek, Model ELx808, Agilent Technologies, and Wilmington, DE, USA). The percentage of inhibition free radicals by each extract was calculated using the equation given below:

$$\% \text{ scavenging of DPPH} = (\text{Abs control} - \text{Abs sample}/\text{Abs control}) \times 100 \quad (1)$$

where Abs control is the absorbance of the DPPH solution without extracts. The capacity of the extracts to reduce DPPH was obtained from the standard curve, and the results are expressed as percentage inhibition against extract concentration.

FRAP assay

Ferric reducing antioxidant powder (FRAP) was used to determine the total antioxidant activity of different solvent extracts of CN leaves following methods described by Shui and Leong [12] with some modifications. $FeSO_{4\ was}$ diluted in deionized water. Then warmed FRAP as working solution was added in all sample extracts. After that, it was incubated in the dark for 1hr and the absorbance at 593 nm. Gallic acid was used as a positive control. The total antioxidant ability was expressed as the concentration of $FeSO_4$ standard solution.

Chemical profiling (GC-MS analysis)

GC-MS analysis was performed based on the methods described by Abdul Rahim [13]. The samples were analyzed by using an Agilent 7890A Gas Chromatograph (Agilent Technologies, Wilmington, DE, USA). Briefly, a 1 mg aliquot of an extract was weighed and added into a GC vial. The powder was then mixed with 1 mL of methanol. Next, the 10 µL mixture was injected into the GC-MS system via splitless mode. The GC-MS system was connected to an MS/MS triple quad detector. The HP-5MS GC column with an inner column and film thickness diameter of 0.25 mm x 30 m x 0.25 µm was used for the GC-MS analysis. The initial oven temperature was set at 100 ºC for 2 min before it was increased gradually to the final temperature of 280 ºC within 17 min at a rate of 10 ºC/min. Helium was used as the carrier gas with a rate of 1 mL/min. All samples were analyzed in three replicates. The spectra of the chromatogram peaks were compared against the National Institute of Standards and Technology (NIST) database library to identify the retention time (R_t) index of common primary and secondary metabolites.

Statistic

All the experiments were conducted in triplicates. The values are expressed as the mean ± standard deviation (SD). The statistical analyses were done using SPSS 25.0 windows statistical package software (SPSS Inc., Chicago, IL). Significant difference between extracts were analysed using one way analysis (ANOVA) followed by Tukey's multiple comparison test.

Results and Discussion

Traditionally CN plant was used as alternative medicinal for various cancer diseases. Many previous studies on CN used parts of leaves because they are green (Figure 1) and have high medicinal properties. In this study CN leaves extracts were prepared using solvents based on their increasing order of polarity (methanol, hexane, dichloromethane and aqueous residue). Currently many research were justified by scientific evidence that CN leaf extracts in chloroform, methanol, and water have the potentials to be used for antiproliferative and anticancer activities against various cancer cell lines [14]. Medicinal plant with anticancer properties is based on phytochemical, antioxidant and analysis of active compounds derivatives present in plant extracts [15]. In this experiment, 80% methanol and fractionation based on polarity was used. This method is effectiveness for plant extract because the different solvents and polarities give significance effects on the chemical contents of the extracts that are important to identify the optimal solvents for the extraction of antioxidant compounds from medicinal plants [16]. The crude methanol was mixed with distilled water because water was employed for the extraction and its universal solubility of polar compounds, while methanol was chosen due its ability to extract lower molecular weight polyphenols and its tendency to yield relevant antioxidant and cytotoxic compounds from CN [17]. Whereas the crude methanol extract was fractionated using different solvents to concentrate and enhance the purity of active compounds and remove unwanted interferences [18].

Extraction of yield of CN leaves was carried out using four different solvents. The yields obtained were extracted from a total dry weight of 500g CN leaves. Among the extract, the highest and the lowest yields were obtained by aqueous residue and hexane extracts, respectively (Table 1). The total percentage of all extracts obtained is 89.67% from the dry weight CN. The high yield in aqueous residue was probably due to the high solubility of phytochemical compounds such as

flavonoids and phenolic contents. Phytochemical is important in developing drug and curing health problems. Various types of solvents used in extraction can recover higher extract yield and some bioactive in plant [19]. This study revealed the significant difference in the extract yield obtained by different solvents.

Figure 1. Whole plant of *Clinacanthus nutans*

Table 1. Percentage yield of methanol extracts and fractions of *Clinacanthus nutans* leaves

No.	Solvent Extracts	Dry Weight (g)	Extract Yield (g)	Percentage of Yield (%)
1.	Methanol	500	18.9167	3.6334
2.	Hexane	15	0.1527	1.018
3.	Dichloromethane	15	0.3276	2.184
4.	Aqueous	15	12.426	82.84

Each value is expressed as mean (n=3)

Phenolics and flavonoids are secondary metabolites and are widely distributed in various plants [20, 21, 22]. Secondary metabolites from medicinal plant were extracted using different solvents. Solvents widely used for extractions are water, ethanol, methanol, acetone and solvent mixtures of different ratios with water, with or without acid. Phenolic constituents and secondary metabolites of plant extraction are based on solvents and its polarity. Phenolic plays a role as primary antioxidant and free radical inhibitors. Whereas flavonoids are polyphenolic compound from numerous phenolics and widely spread in the plant kingdom. Previous studies reported the function of flavonoids such as UV protection, disease resistance, pigmentation and nitrogen fixation simulation in the nodules [23]. This study on phytochemical analysis focuses on the content of flavonoids (TFC) and phenolics (TPC) content (Table 2). The result found that the highest contents of TFC and TPC were in aqueous residue and lower content of TFC and TPC were found in is dichloromethane fractions. Nevertheless, it is reported that the biological properties depend not only on the total extract yield but also on its phytochemical composition [19].

Table 2. Total flavonoids and phenolic content of *Clinacanthus nutans* leaves different solvent extracts and fractions. Mean±SD. (n=3)

Solvents Extracts	Total Flavonoid Content (mgQUE/g)	Total Phenolic Content (mgGAE/g)
Methanol	14.229±0.055	562.784±0.012
Hexane	33.735±0.337	327.548±0.049
Dichloromethane	9.663±0.072	97.191±0.059
Aqueous	27.503±0.089	628.959±0.026

QE-quercitine equivalents; GAE-gallic acid equivalents. Data represented as means ± standard deviation (SD) of replicates (n=3)

Antioxidant molecules were reported in several medicinal plants beneficial in the treatment of several human disease [19] and has the ability to prevent oxidative stress. In the human body, the cell damage caused increasing level of free radical which correlates with and causes various diseases. The free radical was neutralized through antioxidant function [24, 25]. The antioxidant activity of CN could explain its wide-ranging reported bioactivities such as antidiabetic, anticancer, anti-inflammatory and wound healing activities [26]. In this study the antioxidant effects were determined by DPPH and FRAP assays. Both assays involved concentration from 0.031 to 1.0 mg/mL and the significance difference ($p<0.05$) between all the concentrations was observed. The evaluation of DPPH radical scavenging activity inhibition was higher in the crude methanol extract and the lowest was hexane factions (Figure 2). While the FRAP assay resulted clearly in the same result whereby crude methanol extract was higher and the hexane fractions were lower (Figure 3) and this may be due to various kinds of antioxidants present in the samples which react differently with the radical used. Every method has its own advantages and limitations in terms of cost, availability of chemicals, tediousness, preparation time, reproducibility and others [27]. The 1 mg/mL of CN showed the highest percentage of DPPH scavenging activity of crude methanol extract, 51.83±0.010 % followed by dichloromethane fraction (32.43±0.58%), aqueous residue (21.03±0.23%) and hexane fractions (17.38±0.23%).

The result obtained in the previous study by Ismail et al. [28], showed higher hexane fractions percentage of DPPH scavenging values (1530±3.74%) followed by dichloromethane (1039±0.87%), aqueous residue (744.30±8.45%) and lower was crude methanol (560.50±2.45%). It showed different result because the antioxidant activity can be associated with a deficient amount of nitrogen level of the soil level of moisture at different location [29]. The second most commonly used method in the determination of antioxidant is ferric reducing antioxidant power (FRAP) assay [30]. FRAP is an indicator of their potential to confirm antioxidant activity based on the reaction which measures reduction of ferric to ferrous and increase in the absorbance of the reaction mixture that indicates an increase in the reduction power [31]. Standard control of DPPH and FRAP assay were used trolox and gallic, respectively. Thus, the comparison of DPPH and FRAP activity was difficult due to different control standard was used gallic acid and trolox [31, 32, 33]. The solvent effect is an important parameter for the chemical behaviour of antioxidant compounds [30].

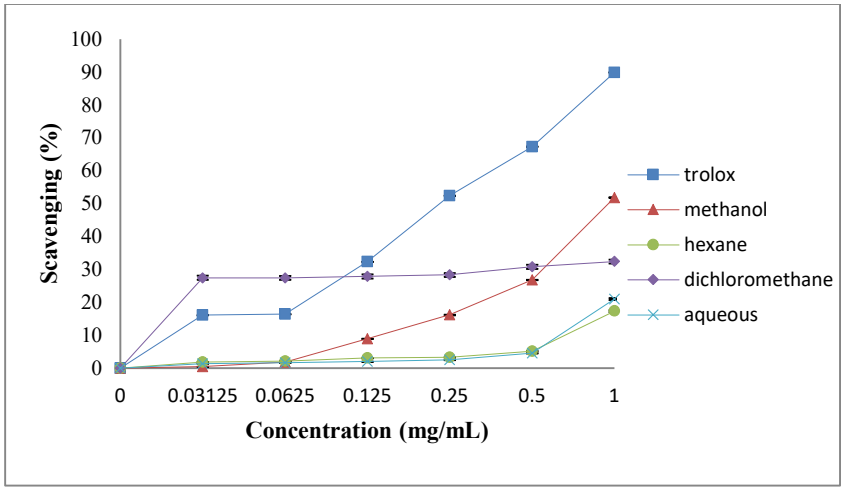

Figure 2. DPPH free radical scavenging activities of various solvent extracts of *Clinacanthus nutans* leaves and trolox

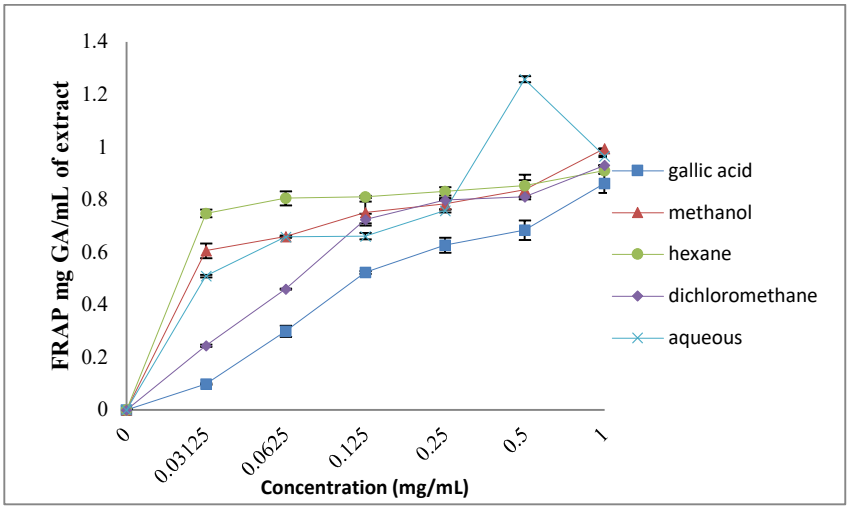

Figure 3. Ferric Reducing Antioxidant Power (FRAP) of the extracts

The phytochemical screening using GCMS found 43compounds presence with various biological activities in different solvent extracts (Table 3). The highest compounds found in hexane fractions were 23 followed by dichloromethane (16), methanol (5) and aqueous fractions (3). From this investigation most of these major compounds are known to exhibit various pharmacological activities. From the investigations methanol extracts contain sugar group such as methylgalactoside and galactoside and also contains fatty acid ester from lineloic acid group and organosulfur. The hexane fractions have the highest phytochemicals, 23 compounds from fatty acid ester (linoleic ester, palmitic acid, methyl ester), terpenoids (diterpene) and alkene. In dichloromethane fractions through the GC-MS analysis the presence of fatty acid ester (linoleic acid, methyl ester), organosulfur, phytosterols/steroid, terpenoids, triterpene and amide were found. In aqueous fractions the phytochemical compounds are present only alkene and diamine group. From these results the variety of phytochemical compounds group is highly present in dichloromethane fractions. The compounds of α and β-Amyrin from triterpene group present in dichloromethane fractions in this experiment are known to possess antidiabetic, anti-inflammatory, antiarthritic and anticancer activities [19, 34]. Amyrins can be found in various plants and plant material such as leaves, bark, wood and resins [35].

Table 3. Phytochemical compounds identified and biological activities of different solvents and fractions of *Clinacanthus nutans* leaves by GC-MS

Retention Time (minutes)	Compound Name	Peak Area (%)[a]				Compound Nature	Biological Activities
		Methanol	Hexane	Dichloromethane	Aqueous		
14.058	Methyl.beta-d-galactopyranoside	29.17	ND	ND	ND	Sugar	Antioxidant, antibacterial
14.058	.alpha-D-Galactopyranoside, methyl	29.17	ND	ND	ND	Sugar	Antimicrobial, antinociceptive cytotoxicity
14.058	.beta.-D-glucopyranoside, methyl	29.17	ND	ND	ND	Sugar	Antimicrobial and antibacterial
18.894	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	ND	0.39	ND	ND	Fatty acid ester	Antimicrobial activity and hypoglycemic effect
18.894	Phthalic acid, butyl isohexyl ester	ND	0.39	ND	ND	Fatty acid ester	Antifungal
20.024	Hexadecanoic acid, methyl ester	0.93	21.03	3.40	ND	Fatty acid ester	Antioxidant, flavour, hypocholesterolemic, nematicide, antiandrogenic, cancer preventive
20.024	Pentadecanoic acid, 14-ethyl-, methyl ester	ND	21.03	3.40	ND	Fatty acid ester	Antibacterial
20.277	Diphenyl sulfone	0.70	ND	0.36	ND	Organosulfur	Antimicrobial, Antibacterial and antifungal
20.53	Benzenepropanoic acid, 3,5-bis(dimethylethyl)-4-hydroxy-, methyl	ND	0.11	ND	ND	Fatty acid	Antifungal , antioxidant
21.593	n-Hexadecanoic acid	ND	0.10	ND	ND	Palmitic acid (fatty acid)	Antifugal, antioxidant, hypocholesterolemic, nematicide, anti-androgenic flavour, haemolytic, 5-Alpha reductase inhibitor, potent antimicrobial agent, antimalarial and antifungal
22.555	Hexadecanoic acid, 15-methyl-, methyl ester	ND	0.48	ND	ND	Fatty acid ester	Antioxidant, nematicide, pesticide, flavour and antiandrogenic
22.555	Heptadecanoic acid, methyl ester	ND	0.48	ND	ND	Fatty acid ester	Antioxidant

24.849	9,12-Octadecadienoic acid, methyl ester	ND	ND	0.96	ND	Fatty acid ester	Antiinflammatory, hypocholesterolemic, cancer preventive, nematicide, hepatoprotective, insectifuge, antihistaminic, antieczemic, antiacne, antiandrogenic, antiarthritic, anticoronary
24.849	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	ND	ND	0.96	ND	Fatty acid ester	Antimicrobial activity, antiinflammatory, hypercholesterolemic, cancer preventive
24.849	9,15-Octadecadienoic acid, methyl ester,(Z,Z)-	ND	ND	0.96	ND	Fatty acid ester	Antimicrobial and anticancer
25.001	9,12-Octadecadienoic acid, methyl ester	ND	10.45	ND	ND	Fatty acid ester	Antiinflammatory, hypocholesterolemic, cancer preventive,nematicide, hepatoprotective, insectifuge, antihistaminic, antieczemic, antiacne, antiandrogenic, anthiarthritic, anticoronary
25.001	9,12-Octadecadienoic acid (Z,Z)-methyl ester	ND	10.45	ND	ND	Fatty acid ester	Anti-inflammatory, hypocholesterolemic, cancer preventive, hepatoprotective, nematicide, insectifuge, antihistaminic, antieczemic, antiacne, 5-alpha reductase inhibitor antiandrogenic, antiarthritic, anticoronary
25.001	10,13-Octadecadienoic acid, methyl ester	ND	10.45	ND	ND	Fatty acid ester	Anticancer activity, antitumor, in vitro cytotoxicity, antioxidant
25.119	9,12,15-Octadecatrienoic acid, methyl ester	ND	ND	3.58	ND	Fatty acid ester	Antiinflammatory, hypocholesterolemic and antiarthritic, antioxidant and antimicrobial
25.119	9,12,15-Octadecatrien-1-ol,(Z,Z,Z)-	ND	ND	3.58	ND	Fatty acid ester	Antioxidant and antibacterial properties
25.119	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)	ND	ND	3.58	ND	Fatty acid ester	Antiinflammatory, hypocholesterolemic and antiarthritic cancer-preventive, hepatoprotective, antioxidant and hypocholesterolemic
25.439	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	ND	41.89	ND	ND	Fatty acid ester	Antiinflammatory, hypocholesterolemic and antiarthritic cancer-preventive, hepatoprotective, antioxidant, antimicrobial ,hypocholesterolemic
25.439	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-cis,cis,cis-7,10,13-Hexadecatriena	ND	41.89	ND	ND	Fatty acid ester	Antiadrogenic
25.692	Phytol	ND	3.36	ND	ND	Terpenoid (diterpene)	Antifungal, stimulant ,antimalarial

26.249	Octadecanoic acid, methyl ester	ND	9.01	1.25	ND	Fatty acid methyl ester	Antiinflammatory, hypocholesterolemic and antiarthritic, antimicrobial and anticancer
27.059	9,12,15-Octadecatrienoic acid,(Z,Z,Z)-	ND	4.88	ND	ND	Fatty acid	Anticancer, antibacterial, antioxidant, antipyretic, cardioprotective, neural function, antiandrogenic and antiarthritic
27.059	9,12,15-Octadecatrienoic acid, ethyl ester,(Z,Z,Z)-	ND	4.88	ND	ND	Fatty acid ester	Antimicrobial, antioxidant and anticancer
27.059	9,12,15-Octadecatrienal	ND	4.88	ND	ND	Alkene	Antibacterial and antioxidant
39.188	.gamma.-Sitosterol	ND	ND	14.21	ND	Steroid	Antidiabetic, antioxidant , antibacterial and prophylactic activities
39.188	Stigmasterol, 22,23-dihydro-	ND	ND	14.21	ND	Terpenoid	Anticancer, antioxidant, hypoglycemic, hypocholesterolemic, antiviral
39.188	.beta.-Sitosterol	ND	ND	14.21	ND	Steroids	Antioxidant, antimicrobial, angiogenic, immunomodulator, antidiabetic, antiinflammatory, anticancer and antinociceptive
39.593	Bicyclo[10.1.0]tridec-1-ene	ND	0.16	ND	ND	Fatty acid (Linoleic acid)	Antioxidant
42.275	.beta.-Amyrin	ND	ND	7.36	ND	Triterpene	Analgesic, antiinflammatory, anticonvulsant, antidepressive, gastroprotective, hepatoprotective, antipancreatic, anticholytic, antihyperglycemic and hypolipidemic effects
42.275	.alpha.-Amyrin	ND	ND	7.36	ND	Triterpene	Analgesic, antiinflammatory, anticonvulsant, antidepressive, gastroprotective, hepatoprotective, antipancreatic, anticholytic, antihyperglycemic and hypolipidemic effects
42.663	Docosanoic acid, methyl ester	ND	0.18	ND	ND	Fatty acid ester	Antimicrobial and antifungal
42.663	Octadecanoic acid, 11-methyl-, methyl ester	ND	0.18	ND	ND	Fatty acid ester	Anticancer
42.933	1,2-Benzenedicarboxylic acid, mono (2-ethylhexyl) ester	ND	0.65	ND	ND	Fatty acid ester	Antimicrobial, antiinflammatory, antioxidant, hypoglycemic effect and anticancer
44.738	9,12-Octadecadienoic acid (Z,Z)-2,3-dihydroxypropyl ester	ND	0.31	ND	ND	Fatty acid ester	Antiatherosclerotic, antiinflammatrory, analgesic, anthirheumatic and antioxidant

44.738	9,12-Octadecadienoic acid (Z,Z)-2-hydroxy-1-(hydroxymethyl)ethyl ester	ND	0.31	ND	ND	Fatty acid ester	Hypcholesterolemic, nematicide, anthiarthritic, hepatoprotective , antiandrogenic, hypcholesterolemic 5-alpha reductase inhibitor, antihistaminic, anticoronory, insectifuge, antieczema and antiacne
45.683	9- Octadecenamide, (Z)-	ND	ND	1.79	ND	Amide	Antifungal and antibacterial
45.936	Cyclotrisiloxane, hexamethyl-	ND	ND	ND	100	Alkane	Antimicrobial, antioxidant and antibacterial
45.936	Tetrasiloxane, decamethyl	ND	ND	ND	100	Alkane	Antibacterial and antifungal
45.936	1,2-Propanediamine	ND	ND	ND	100	Diamine	Antimicrobial

*ND= not detected

CN has been reported to have various phenolic, terpenoids and some other bioactive compounds such as benzenoids, cerbrosides, glycoglycerides, fatty acids, chlorophyll derivatives, phytosterols and sulphur containing glucosides that contribute to its diverse bioactivities [36]. Phytol that is present in hexane fractions, is an important terpenoid or diterpene that possesses antimicrobial, antioxidant and anticancer activities [19, 37, 38, 39]. Terpenoids and alkaloids compound have also been reported to have potent activity against gastric ulcers and relax cardiovascular muscle [40]. The terpenoids are widely distributed in nature and are found in higher plants. They represent the second largest class of secondary metabolites with more active constituents. It is now known that terpenes with a pleasant aroma are extracted from the vegetable or plants containing them and some are precursors of vitamins and insecticides [35, 41]. The various phenolic and fatty acid compounds were found in ethanol and ethyl acetate leaf extract of CN [26, 42]. Phenolics compounds esters, alkanes, aldehydes, alkenes and ketones are the other major volatile compounds which have antiulcer, anti-inflammatory, antiarthritic, antidiabetic, hypolipidemic and cytotoxic activities [40, 43]. Plant phenolics are one of the most abundant vital group of phytochemicals and are widely known for their antioxidant and radical scavenging activity with potential impacts on human health. The phenolics are natural antioxidants which can control oxidative stress related degenerative disease. The adverse effects of oxidative stress have been found to be controlled by the antioxidant activities of this group of bioactive compounds. [6]. The compounds have attracted great attention recently due to their potential to neutralize reactive oxygen species and other free radicals. Phenolic and flavonoid compounds display antitumor, antiadhesive, antimicrobial and anti-inflammatory properties and protection against chronic disease by the reduction of oxidative stress solely or synergistically with other phenolic containing amalgams [25]. In this study the various phenolics group was found in all the solvent and different extracts tested on CN extracts. Thus, it is shown that all the solvent extractions important roles as antioxidant are widely distributed in plants and possess ultraviolet protection, pigmentation and disease resistance [9].

Ester is a major compounds and origin from phenolics groups which shown presence in this all solvent extraction through GCMS analysis. Fatty acid is one of the groups of esters. Previous study on ethanol and ethyl acetate extract of CN indicated the presence of ester group compounds such as palmitic acid, myristic acid, linoleic acid or omega 3 fatty acid, stearic acid and oleic acid [26]. In this study the presence of ester group of linoleic acid in hexane fractions and dichloromethane fractions was shown while palmitic acid found in hexane fractions. Fatty acid methyl ester was found in hexane and dichloromethane fraction at a retention time of 26.249 minutes with percentage peak area for hexane and dichloromethane 9.01% and 1.25%, respectively. The methyl ester, stearic acid or stearate is a saturated 19-carbon-chained compound also known as octadecanoic acid methyl ester was reported to have antimicrobial, antiviral, potential anticancer properties [44], anti-inflammatory, hypocholesterolaemia, antiarthritic and antimicrobial [44, 45, 46]. In addition, phytochemical compounds found from GC-MS were not only phenolic and flavonoid contents but also consist of many other compounds such as diterpene, aromatic methyl esters, fatty acid, ester, fatty acid, vitamin and so on which were compared by NIST mass spectra l library [29]. Previous study of CN extract leaf oven and air-dried leaves demonstrated that the CN exhibited better antioxidant with the presence of phenolics, terpenoids and sulphur containing glucosidase compounds [26, 47]. Glucosidase is one of the sugar group that present in this study such as methygalactosidase and galactosidase that are present in methanol crude extract which has potential biological activity such as antioxidant and antibacterial [48, 49, 50]. Organosulfur is also present in dichloromethane fractions for antimicrobial, antibacterial and antifungal potential [51].

In this study the method of extraction involved the proses of fractionated on the crude extracts of CN leaves. The fractionated crude extracts used polar and nonpolar solvents that obtain phytoconstituent which rich of biological active standardize fractions [6]. Previous study indicated the effect of phytochemical compounds present in leaves CN is caused by first step method involved the crude extraction with macerating

the leaves with methanol to water (4:1) at room temperature. This method involved lower temperature in order to avoid degradation of compounds to produce polyphenols which produce many biological compounds functions [29, 51]. This extraction method has ability to produce biological activities profiling.

Conclusion

The greenly leaves of CN aqueous fractions and crude methanol extracts from different solvent extracts exhibited higher yield percentage of extracts and high values phytochemicals of flavonoid and phenolic contents. In addition, antioxidant study of DPPH radical scavenging and FRAP activity indicated the highest result was methanol crude extract and dichloromethane fractions may be explored as new sources of antioxidants in medicinal plant research. However, from the GCMS analysis, it is found that the dichloromethane fractions indicated a variety of bioactive compounds and medicinal properties. Furthermore, cytotoxicity study of the antioxidant and phytochemicals rich fractions is necessary to verify their suitability for future pharmaceutical applications.

Acknowledgement

This work was supported and funded by a FRGS grant (203/CIPPT/6711340) from the Ministry of Higher Education, Government of Malaysia and a USM Bridging Grant (304/CIPPT/6316239) to Hasni Arsad.

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