Sains Malaysiana 52(1)(2023): 211-222
http://doi.org/10.17576/jsm-2023-5201-17

A Comparative Study of Anti-Inflammatory Properties and Activities of Green and Red *Christia vespertilionis* Leaves

(Kajian Perbandingan Sifat dan Aktiviti Anti-Keradangan Daun *Christia vespertilionis* Hijau dan Merah)

IZZAH FARHAH ZAMBARI[1], NUR AIRINA MUHAMAD[1,*] & SITTI RAHMA ABDUL HAFID[2]

[1]*Institute of Biological Sciences, Faculty of Science, Universiti Malaya, 50603 Kuala Lumpur, Federal Territory, Malaysia*
[2]*Nutrition Unit, Product Development and Advisory Services Division, Malaysian Palm Oil Board, 43000 Kajang, Selangor Darul Ehsan, Malaysia*

Received: 27 May 2021/Accepted: 5 October 2022

ABSTRACT

Christia vespertilionis is a plant traditionally used to treat various diseases, including inflammation. This study aims to compare and analyze the anti-inflammatory properties and activities of green and red *C. vespertilionis* leaves and to determine the correlation between the protease inhibition assay and phenolic content. Preliminary phytochemical analysis showed the presence of alkaloids, tannins, saponins, cardenolides, phenolics, cardiac glycosides, flavonoids, and steroids in green and red leaves. Green leaf had the highest anti-inflammation potential, with a high extraction yield (6.39 ± 0.01%), phenolic content (29.25 ± 0.50 mg) gallic acid equivalent [GAE]/mL), flavonoid content (1.57 ± 0.03 mg quercetin acid equivalent [QE]/mL) and tannin content (22.70 ± 3.15 mg tannic acid equivalent [TAE]/mL). Green leaf contained more anti-inflammatory compounds, including n-hexadecanoic acid, phytol, 9, 12, 15-octadecatrienoic acid (Z, Z, Z)- and squalene. The protease inhibition assay showed that green leaf had anti-inflammatory activity at 66.55 ± 2.59% with the half-maximal inhibitory concentration (IC_{50}) of 284.59 ± 10.63 µg/mL. The green leaf showed a significant positive correlation between the protease inhibition assay and phenolic content, with $R^2 = 0.6821$. We conclude that both green and red *C. vespertilionis* types have the potential to act as anti-inflammatory agents, but green leaves are preferable.

Keywords: Anti-inflammatory; *Christia vespertilonis*; green; phytochemical compounds; red

ABSTRAK

Christia vespertilionis ialah salah satu tanaman yang digunakan secara tradisi untuk merawat pelbagai penyakit termasuk keradangan. Kajian ini dilakukan untuk membandingkan dan menganalisis sifat dan aktiviti anti-keradangan ekstrak daun *C. vespertilionis* hijau dan merah serta untuk mengkaji hubungan antara ujian perencatan protease dan kandungan fenol. Analisis fitokimia awal menunjukkan adanya alkaloid, tanin, saponin, kardenolid, fenol, kardiak glikosida, flavonoid dan steroid pada daun hijau dan merah. Daun hijau dikenal pasti sebagai yang paling berkesan untuk potensi anti-keradangan dan menunjukkan hasil pengekstrakan tinggi (6.39 ± 0.01%), kandungan fenol (29.25 ± 0.50 mg asid galik setara [GAE]/mL), kandungan flavonoid (1.57 ± 0.03 mg asid kuersetin setara [QE]/mL) dan kandungan tanin (22.70 ± 3.15 mg asid tanik setara [TAE]/mL). Daun hijau mengandungi lebih banyak sebatian anti-keradangan, iaitu asid n-heksadekanoik, fitol, 9, 12, asid 15-oktadekatrienoik, (Z, Z, Z)- dan squalene. Daun hijau juga menunjukkan aktiviti anti-keradangan yang tinggi seperti yang ditunjukkan oleh ujian perencatan protease iaitu 66.55 ± 2.59% dengan nilai kepekatan perencatan separuh maksimum (IC_{50}) iaitu 284.59 ± 10.63 µg/mL. Hubungan positif yang signifikan antara ujian perencatan protease dan kandungan fenol pada daun hijau menunjukkan $R^2 = 0.6821$. Kesimpulannya kedua-dua jenis daun, *C. vespertilionis* hijau dan merah berpotensi bertindak sebagai agen anti-keradangan, tetapi daun hijau menunjukkan hasil yang lebih baik.

Kata kunci: Anti-keradangan; *Christia vespertilonis*; hijau; merah; sebatian fitokimia

INTRODUCTION

Inflammation has become a prevalent disease and has been the leading cause of death if not cured early. Three out of five people die from chronic inflammatory diseases such as stroke, chronic respiratory disease, heart disorders, cancer, obesity, and diabetes worldwide (de

Barcelos, Troxell & Graves 2019; Deepak, Axelrad & Ananthakrishnan 2019; Tsai et al. 2019). Inflammation is an infection or irritation of living cells or tissue produced by injury and the body's response to it (Amazu et al. 2010). The injured tissue increases vascular permeability, enhances protein denaturation, and builds membrane alteration. The chemotaxis mechanism causes the body to release kinins, prostaglandins, and histamine as a natural defense (Ruiz-Ruiz et al. 2017). Inflammatory reactions reduce tissue destruction through scavenging reactive oxygen species known as activate matrix metalloproteinase (Cotran, Kumar & Robbins 1994). Nonsteroidal anti-inflammatory drugs (NSAIDs) and steroids are currently used in clinical settings to treat inflammation. However, these drugs have adverse side effects such as gastric intestinal mucosa and heart and kidney disorders (De Groot & Scott 2007). Furthermore, drugs such as aspirin and steroids are only effective in relieving symptoms after a single dose (Debnath et al. 2013).

Plant-derived drugs have been used as medicine for centuries. Phytochemical properties of over 250,000-500,000 plant species have been investigated worldwide (Habila et al. 2011). A medicinal plant is a choice as a therapeutic drug due to the abundance of active compounds, such as alkaloids, phenols, and secondary metabolites. Bioactive compounds in plants are essential for human health to combat diseases and have been studied extensively by researchers. Many pharmaceutical companies are now focusing on plant-derived drug development, which is gaining popularity worldwide because of its affordability, convenience, and safety (Apu et al. 2012; Burke, Smyth & FitzGerald 2005). It has various biological activities, as well as unique chemical and pharmacological properties. Plant-derived drugs that contain secondary metabolites with multiple biological activities can be used to treat a wide range of diseases.

Christia vespertilionis, or butterfly wing, has a promising future of becoming an anti-inflammatory agent among the various medicinal plants worldwide. This plant originates from South-eastern China, India, Thailand, Cambodia, Laos, Vietnam, Indonesia, and Malaysia. It is commonly called 'Daun rama-rama' due to the leaf structure similar to butterfly wings in Malaysia. It has anti-cancer, anti-inflammatory, anti-proliferative, and anti-plasmodial properties (Hofer et al. 2013; Nguyen-Pouplin et al. 2007; Osman et al. 2017). This plant has two leaf colours, one with green leaves and the other with red leaves. Local residents use it to treat tuberculosis, scabies, snake bites, bronchitis, and poor blood circulation (Garnock-Jones 1983; Whiting 2007). Research has shown that this plant has anti-inflammatory (Nguyen-Pouplin et al. 2007; Osman et al. 2017; Rayburn, Ezell & Zhang 2009), antioxidant (Lee et al. 2020), anti-cancer, antidiabetic (Murugesu et al. 2020), antiplasmodial (Upadhyay et al. 2013), and anti-proliferative (Hofer et al. 2013) properties.

In Malaysia, cancer patients widely consume *C. vespertilionis* and have gained the attention of researchers to discover its medicinal potential. The previous study has shown that *C. vespertilionis* contains bioactive secondary compounds such as phenols, alkaloids, triterpenes, fatty acids, and long-chain alcohols (Hofer et al. 2013). Additionally, *C. vespertilionis* roots contain flavonoids, coumarins, and quinones, leading to significant results for anti-breast cancer and antioxidants, while the aerial part of the plant comprises antibacterial compounds, namely corynoxidine and palmatine (Lee et al. 2020; Nguyen-Pouplin et al. 2007; Sharma & Cannoo 2016). Christene and christanoate are two recently reported anti-plasmodial agents isolated from *C. vespertilionis* (Upadhyay et al. 2013). Lee et al. (2020) discovered that the *C. vespertilionis* root has anti-cancer properties against human breast carcinoma cell lines (MCF-7 and MDA-MB-231). Hofer et al. (2013) also stated that *C. vespertilionis* has an anti-proliferative activity against medullary thyroid carcinoma (MTC) and small intestinal neuroendocrine tumors (SI-NET) cell lines. Few studies are available on phytochemical screening of *C. vespertilionis* with both types of leaves (green and red), focusing on anti-inflammatory properties and activities. Thus, this research aims to compare and analyze the anti-inflammatory properties and activities of green and red *C. vespertilionis* leaves and determine the correlation between total phenolic content and protease inhibition assay.

Materials and Methods

Chemicals and Reagents

Analytical grade ethanol was purchased from EAM (Selangor, Malaysia) for plant extraction. For phytochemical analysis, the analytical grade of all chemicals and reagents was acquired from EAM (Selangor, Malaysia), R&M Chemicals (Selangor, Malaysia), and Sigma Aldrich (St. Louis, Missouri, USA).

Plant Material

Green and red *C. vespertilionis* leaves were collected from Floranika Nursery Sungai Buloh, Selangor

(Malaysia) (located at the latitude and longitude of 3° 13' 6.7764'' N, 101° 34' 18.1704'' E). The voucher specimen was certified by Dr. Yong Kien Thai from Plant Taxonomy, Rimba Ilmu, University of Malaya (UM). The voucher specimen of green *C. vespertilionis* (KLU 50026) and red *C. vespertilionis* (KLU 50025) were placed at the herbarium, UM. Each type of leaf (green and red) were weighed separately at 20 g and air-dried for a week. The dried leaves were ground into a powder using a blender and kept in a closed container until further use.

SAMPLE EXTRACTION

A total of 1 g of dried powdered sample for each type of *C. vespertilionis* leaves were extracted separately with 200 mL of ethanol using the Soxhlet apparatus for 8 h. The extract was filtered and evaporated using a rotary evaporator, then filtered to remove any particles and kept in microcentrifuge tubes at 4 °C for further use. A 1 mL extract was air-dried to determine its dry weight. The extraction yield of each extract was calculated using the following formula:

$$\text{Extraction yield (\%)} = \frac{m^2}{m^1} \times 100$$

where m^1 is the mass of dry weight of *C. vespertilionis* leaves, (g); and m^2 is the mass of crude extract of *C. vespertilionis* leaves, (g).

QUALITATIVE ANALYSIS

Qualitative analysis was carried out to confirm the presence of compounds in the extracts using standard analytical techniques such as alkaloid (Mayer's test), tannin ($FeCl_3$ test), saponin (foam test), cardenolide (sodium picrate test), phenolic ($FeCl_3$ test), cardiac glycoside (Keller-Kiliani test), flavonoid (ammonia test), steroid (Libermann-Burchard test) and terpenoid (Salkowski test) from both green and red *C. vespertilionis* leaf extract.

Alkaloid test
A 1.0 mL of 1% (v/v) hydrochloric acid (HCl) was added to 3.0 mL of extract. After 20 min of heating, the mixture was cooled and filtered. After adding two drops of Mayer's reagent, a creamy precipitate indicated the presence of an alkaloid in the extract.

Tannin test
A 1.0 mL of 10% (w/v) ethanolic potassium hydroxide (KOH) was added to 1.0 mL of extract. A dirty white precipitate indicated the presence of tannin.

Saponin test
A 2.0 mL extract was shaken vigorously with 2.0 mL of water for 2 min before being warmed. Frothing indicated the presence of saponin.

Cardenolide test
A 2.0 mL of glacial acetic acid containing one drop of 5% (w/v) iron (III) chloride solution ($FeCl_3$) was added to 1.0 mL of extract. Then, it was followed by 2.0 mL of concentrated sulphuric acid (conc. H_2SO_4). A brown ring at the interface indicated the presence of a deoxy sugar characteristic of cardenolide.

Phenolic test
Two drops of 5% (w/v) $FeCl_3$ was added to 1.0 mL of extract. A greenish precipitate indicated the presence of phenolic.

Cardiac glycoside test
A 2.0 mL of chloroform and 2.0 mL of conc. H_2SO_4 was added to 1.0 mL of extract. A reddish-brown colour at the interface indicated the presence of an aglycone portion of cardiac glycoside.

Flavonoid test
A 1.0 mL of 10% (w/v) sodium hydroxide (NaOH) was added to 3.0 mL extract. A yellow colouration indicated the presence of flavonoids.

Steroid test
Five drops of conc. H_2SO_4 was added to 1.0 mL of extract. Red colouration indicated the presence of steroids.

Terpenoid
A 0.5 mL chloroform and a few drops of conc. H_2SO_4 was added to 1.0 mL extract. Reddish brown precipitate indicated the presence of terpenoids.

QUANTITATIVE ANALYSIS

Total phenolic, flavonoid, and tannin contents were quantified in green and red *C. vespertilionis* leaf extracts separately. Gallic acid and quercetin were used as standard anti-inflammatory agents, according to Mokhtar et al. (2019), with some modifications to the Folin-Ciocalteu method. Meanwhile, tannic acid was used as a standard anti-inflammatory agent according to the method described by Folin-Ciocalteu, as modified by

Mesfin and Won (2019). The absorbance of extracts was measured using a Shimadzu UV-1700 spectrophotometer (Tokyo, Japan).

Total phenolic content

About 600 µL of Folin's reagent with 1.0 mL of 7.5% (w/v) sodium carbonate (Na_2CO_3) was added into 200 µL of extract. The mixture was incubated in the dark for 2 h. Then, the absorbance was measured at 765 nm against gallic acid dilutions (0.0-1.0 mg/mL) as the standard solution. The total phenolic content was expressed in terms of gallic acid in mg GAE/mL of extract.

Total flavonoid content

A total of 600 µL of methanol (CH_3OH), 40 µL of 10% (w/v) aluminum chloride ($AlCl_3$), 40 µL of 1 M potassium acetate (CH_3COOK), and 1.12 mL of Milli-Q water were mixed and added into 200 µL extract, then, incubated for 30 min at room temperature. The absorbance was measured at 420 nm and compared to the standard solution of quercetin dilutions (0.0-1.0 mg/mL). The total flavonoid content was expressed in terms of quercetin in mg QE/mL of extract.

Total tannin content

A 250 µL of Folin's reagent, 500 µL of 35% (w/v) Na_2CO_3, and 3.75 mL of distilled water were added to 500 µL of the extract before incubating at room temperature for 30 min. The absorbance was measured at 725 nm against tannic acid dilutions (0.0-1.0 mg/mL) as a standard solution. The total tannin content was expressed in terms of tannic acid in mg TAE/mL of extract.

IN VITRO ANTI-INFLAMMATORY ASSAY VIA PROTEASE INHIBITION ACTIVITY ASSAY

Protease is one of the mediators involved in inflammation by cleaving chemokines and cytokines. The protease inhibition activity assay for *C. vespertilionis* extracts of both types was conducted according to Gunathilake, Ranaweera and Rupasinghe (2018) and Sakat, Juvekar and Gambhire (2010), with some modifications. Protease level was measured during the inflammatory process. A total of 450 µL of 5% (w/v) trypsin was pipetted into 50 µL of green and red leaf extract, separately in different concentrations (100-3000 µg/mL), while 50 µL of aspirin (100-3000 µg/mL) served as a standard. The mixtures were incubated at 37 °C for 10 min. Afterward, 250 µL of 1% (w/v) casein was added and incubated for 20 min. The reaction was terminated by adding 700 µL of 10% (w/v) trichloroacetic acid and centrifuged at 3000 rpm for 5 min at 25 °C. The absorbance of the supernatant was measured at OD_{210nm} using a Shimadzu UV-1700 spectrophotometer (Tokyo, Japan). The assay was carried out in triplicates, and the results were compared to bovine serum albumin. The percentage of inhibition was measured using the formula below:

$$\text{Percentage inhibition (\%)} = \frac{(A^2 - A^1)}{A^2} \times 100$$

where A^1 is the sample absorbance; and A^2 is the control absorbance.

GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS) ANALYSIS

A volume of 1.0 mL for *C. vespertilionis* green and red leaves extracts were diluted to 500 ppm with ethanol into a 1.5 mL vial. Gas chromatography-mass spectrometry (GC-MS) analysis was performed using Shimadzu, GCMS-QP2010 Ultra (Tokyo, Japan). A total of 0.5 µL sample was auto-injected into the system. The system was supplied with a capillary column of RTX5MS with a length × diameter of 30.0 m × 0.25 mm and a 0.25 µm of thickness. The injection temperature was set to 200 °C; injection mode was splitless. The initial temperature was 50 °C (3 min) with an accelerating rate of 10 °C (1 min) to 300 °C (10 min). Helium gas was used, with a linear velocity of 47.8 cm/s. Electron ionization (EI) mode was performed at 70 eV with a spectral range of 35–500 m/z for mass spectra results. The ion source temperature was fixed at 150 °C, and the interface temperature was at 230 °C with a solvent cut-off time of 3 min. The start time was set at 3 min and the final time was 33 min. The total flow programmed was 21.6 mL/min with a column flow of 1.69 mL/min. The compounds were determined based on the interpretation of mass spectrum with standard reference spectral using the National Institute of Standards and Technology Mass Spectral Library 2011 (NIST 2011) version 2.0g databases (https://www.nist.gov/srd/nist-standard-reference-database-1a).

STATISTICAL ANALYSIS

All tests were conducted in triplicates and presented as average ± standard deviation using a two-way analysis of variance (ANOVA) test. The analysis was done using GraphPad Prism (version 8.0.2). Mean values were considered statistically significant when $P < 0.05$, and the correlation of protease inhibition activity assay and total phenolic content of *C. vespertilionis* leaf extracts were determined using Pearson's correlation coefficient;

the difference was considered statistically significant when $P < 0.05$.

Results and Discussion

Extraction Yield

Table 1 shows that Green *C. vespertilionis* leaf extract has an extraction yield of 6.39 ± 0.01%. Meanwhile, red *C. vespertilionis* leaf extract has a low extraction yield of 1.65 ± 0.02%. Extraction was performed using the Soxhlet extraction to discover phytochemical compounds. A previous study showed an extraction yield of 6.40% from *C. vespertilionis* leaf ethanolic extract (Mutalib & Latip 2019), while another study showed that red *C. vespertilionis* leaf has an extraction yield of 2.94% using CO_2 liquid and ethanol as a solvent (Ariff et al. 2018). This study's outcomes are similar to Mutalib and Latip (2019) results, with minor variations. However, the extraction yield of *C. vespertilionis* value can increase when using an appropriate extraction method. According to one study, optimizing the efficient extraction method for the respective plant takes time (Dhanani et al. 2017). Therefore, each plant requires a different extraction method to exhibit the phytochemical compounds.

TABLE 1. Extraction yield in green and red *C. vespertilionis* leaf extract

C. vespertilionis leaf extract	Extraction yield (%)
Green	6.39 ± 0.01[a]
Red	1.65 ± 0.02[a]

All values are triplicate with mean ± SD. a, refer to $P < 0.05$, which is a significant difference between green and red leaf extract, using a two-way ANOVA test

Qualitative Analysis

C. vespertilionis leaf extracts were qualitatively analyzed to identify compounds. This is important as a preliminary test before further quantitative analysis. Table 2 shows green and red leaf compounds, including phenolic, cardiac glycoside, steroid, cardenolide, flavonoid, alkaloid, saponin, and tannin. These compounds participate in many biological activities. It has been reported that phenolics can act as free radical scavengers, antioxidants, anti-inflammatory, and anti-carcinogenic agents (Shahidi & Yeo 2018). Furthermore, it can reduce inflammation in chronic diseases such as cardiovascular disease, cancer, diabetes, and bacterial and parasitic infections (Canini et al. 2007). However, a cardiac glycoside is important for cardiac muscle functioning (Aslam et al. 2009), while steroids and cardenolides are signaling molecules with the potential to alter membrane fluidity (Sadava et al. 2011). Cazarolli et al. (2008) reported that flavonoids reduce free radical damage and oxidative reduction of macromolecules. Lin et al. (1999) stated that flavonoids have anti-inflammatory properties and alkaloids are efficient against pathogens. However, Saponin has anti-fungal, antibacterial, and anti-protozoal activities and inhibits damage to the upper digestive tract (Aslam et al. 2009; Ayoola & Adeyeye 2010), while tannin has antioxidant activity (Rajurkar & Gaikwad 2012).

Quantitative Analysis

The total phenolic, flavonoid, and tannin content of both extracts have been measured to investigate their potential anti-inflammatory properties. Table 3 shows that the total phenolic content for green *C. vespertilionis* leaf extract was 29.25 ± 0.50 mg GAE/mL. Meanwhile, the total flavonoid content was 1.57 ± 0.03 mg QE/mL, and the tannin content of 22.70 ± 3.15 mg TAE/mL. This finding showed similar results as reported by Mutalib and Latip (2019). The total phenolic content was higher than the total flavonoid content in *C. vespertilionis* leaf ethanolic extract. Smitha and Reshma (2019) also detected flavonoids and tannin in *C. vespertilionis* in their study. Lee et al. (2020) discovered phenolic in *C. vespertilionis* root ethyl acetate extract. However, phenolic, flavonoid, and tannin content of red *C. vespertilionis* leaf extract was 24.16 ± 0.50 mg GAE/mL, 1.31 ± 0.04 mg QE/mL, and 17.19 ± 0.32 mg TAE/mL, respectively, which were lower than green leaf extract.

TABLE 2. Phytochemical compounds in green and red *C. vespertilionis* leaf extract

No.	Phytochemical compounds	*C. vespertilionis* leaf extract	
		Green	Red
1	Alkaloid	+	+
2	Tannin	+	+
3	Saponin	+	+
4	Cardenolide	+	+
5	Phenolic	+	+
6	Cardiac glycoside	+	+
7	Flavonoid	+	+
8	Steroid	+	+
9	Terpenoid	-	-

+ present, - absent

TABLE 3. Total phenolic, flavonoid and tannin content in green and red *C. vespertilionis* leaf extract

C. vespertilionis leaf extract	Total phenolic content (mg GAE/mL)	Total flavonoid content (mg QE/mL)	Total tannin content (mg TAE/mL)
Green	29.25 ± 0.50^{a}	1.57 ± 0.03^{a}	22.70 ± 3.15^{ns}
Red	24.16 ± 0.50^{a}	1.31 ± 0.04^{a}	17.19 ± 0.32^{ns}

All values are triplicate with mean ± SD. a, refer to $P < 0.05$, a significant difference between green and red leaf extract, using a two-way ANOVA test, while ns is a non-significant

IN VITRO ANTI-INFLAMMATORY ASSAY VIA PROTEASE INHIBITION ACTIVITY ASSAY

Therefore, another approach was carried out to prove that green *C. vespertilionis* leaf extract contains more phytochemical compounds responsible for anti-inflammation than the red leaf. The extract of both leaves was subjected to *in vitro* anti-inflammatory assay. Protease is one of the many biological mediators that exhibits when inflammation occurs. It regulates the inflammation complex system by cleaving cytokines, chemokines, and immune components. In this test, high protease inhibition indicated that the substance could treat inflammation. Table 4 shows that green *C. vespertilionis* leaf extract inhibited protease with 66.55 ± 2.59% and IC_{50} value of 284.59 ± 10.63 µg/mL, compared to red leaf extract with 62.63 ± 2.49% and IC_{50} value of 226.59 ± 10.35 µg/mL. Only red leaf extract differs significantly from the standard anti-inflammatory drug,

TABLE 4. Protease inhibition activity assay in green and red *C. vespertilionis* leaf extract with aspirin as a control

Sample	Inhibition (%)	IC_{50} value (µg/mL)
Green *C. vespertilionis* leaf extract	66.55 ± 2.59	284.59 ± 10.63[a]
Red *C. vespertilionis* leaf extract	62.63 ± 2.49	226.59 ± 10.35[a,b]
Aspirin	75.66 ± 0.99	275.72 ± 6.93[b]

All values are triplicate with mean ± SD. a and b; each refers to $P < 0.05$, a significant difference between different samples, using a two-way ANOVA test

aspirin, 75.66 ± 0.99%, and IC_{50} value of 275.72 ± 6.93 µg/mL. The difference between green leaf extract and aspirin was 9.11%, indicating that green *C. vespertilionis* leaf extract can inhibit proteases compared to red leaf.

Oyedapo (2001) also stated that phenolic compounds, such as flavonoids and tannins, are primarily responsible for anti-inflammatory activity. Other biological activities of phenolic compounds include antioxidant, antiviral, and anticancer properties (Lee et al. 2020). As mentioned previously, protease plays a role in inflammation. Therefore, the anti-inflammatory activity can be deduced by correlating the protease inhibition assay with the total phenolic content. The analysis showed that the protease inhibition assay with total phenolic content has significant and positive correlations, with green leaf extract having $R^2 = 0.6821$ and red leaf extract having $R^2 = 0.6068$ (Figure 1). The result indicated that the phenolic compounds in *C. vespertilionis* leaf extract, mainly for the green type of leaf, can be the main contributor to the anti-inflammatory activities.

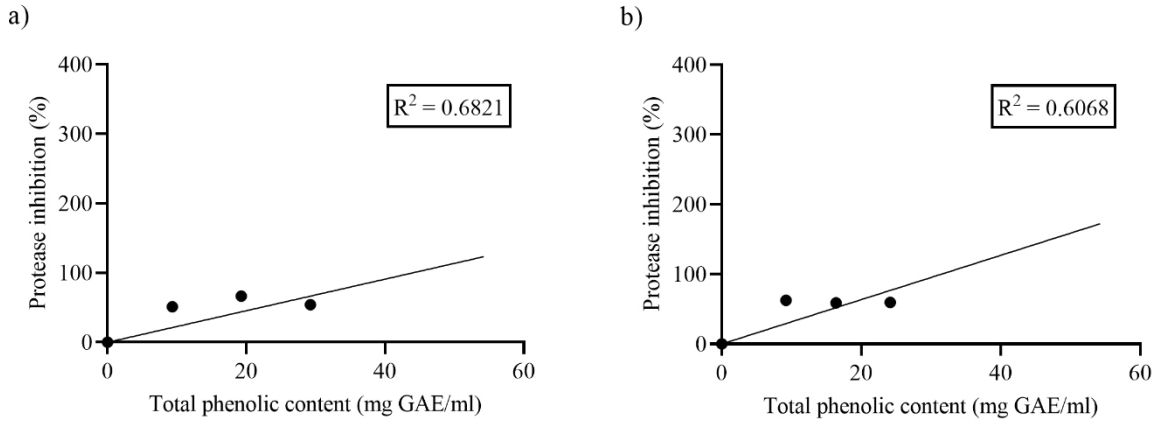


FIGURE 1. Correlation linear regression of protease inhibition (%) versus total phenolic content (mg GAE/mL) of a) green *C. vespertilionis* leaf extract and b) red *C. vespertilionis* leaf extract

GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS) ANALYSIS

Gas chromatography-mass spectrometry (GC-MS) is an important tool for identifying phytochemical compounds from a complex phytochemical mixture using a technique of separation in gas chromatography (GC) system and measuring on mass spectrometry (MS) with electron ionization (EI) ion source. Figure 2 and

Table 5 show that four major peaks greater than 4% (Abd Rahim et al. 2018) were confirmed in green leaf extract as n-hexadecanoic acid or namely palmitic acid, phytol, 9, 12, 15-octadecatrienoic acid (Z, Z, Z)- or known as linolenic acid and squalene. These compounds were also present in red leaf extract, except phytol. This was also reported by Sidek et al. (2019), who discovered similar major compounds in *C. vespertilionis* leaf extract. Mohd Yasin et al. (2020) identified phytol as a major compound in *C. vespertilionis* extract. Compounds of n-hexadecanoic acid (Aparna et al. 2012), phytol (Silva et al. 2014), 9, 12, 15-octadecatrienoic acid, (Z, Z, Z)- (Sermakkani & Thangapandian 2012; Suman, Chakkaravarthi & Elangomathavan 2013) and squalene (Kelly 1999; Lacatusua et al. 2018) have anti-inflammatory activity.

These compounds have additional biological activities. Lacatusua et al. (2018) showed that squalene could reduce skin damage using *Amaranthus cruentus*

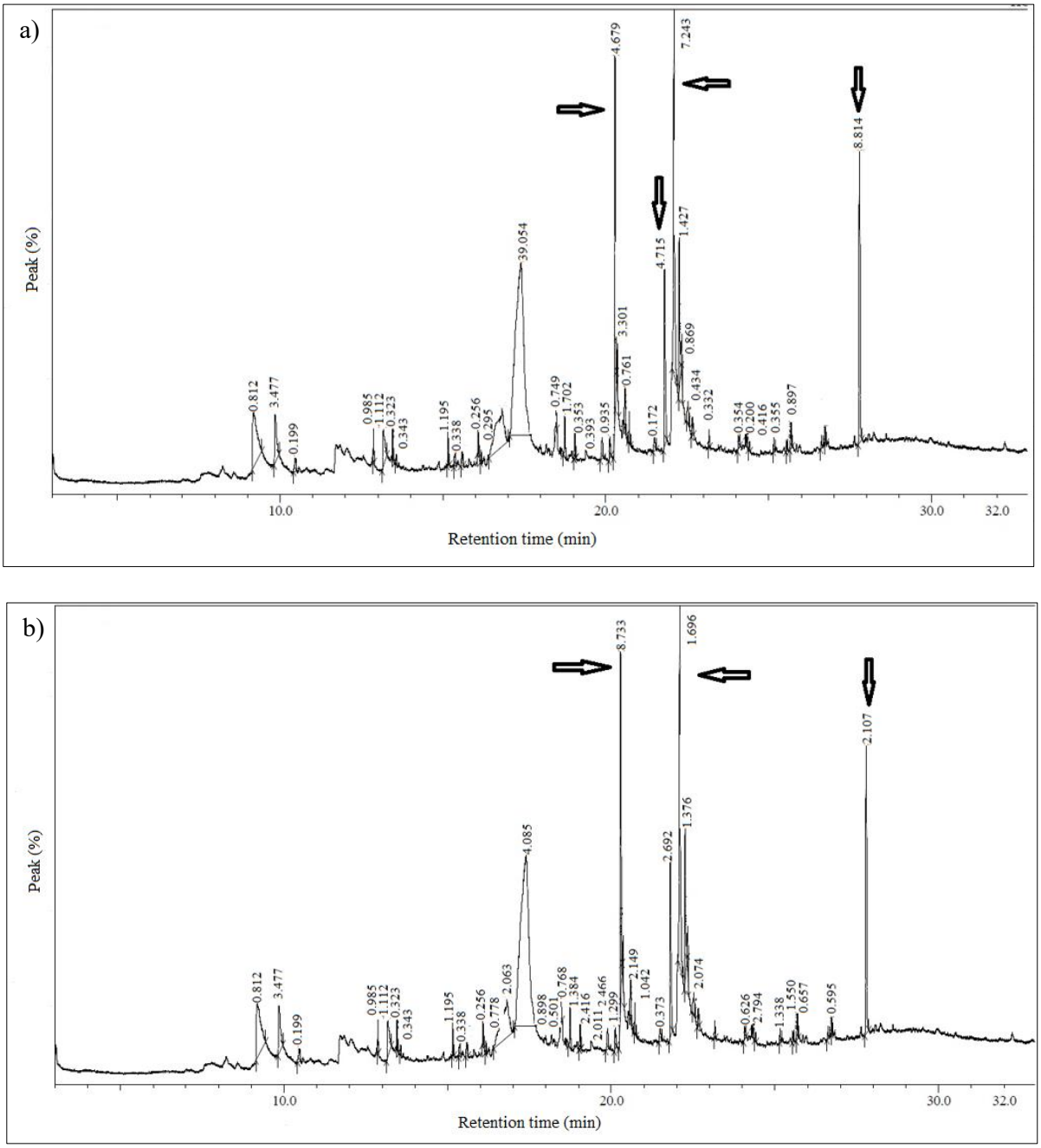
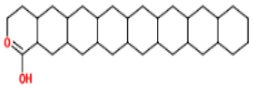
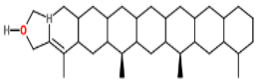
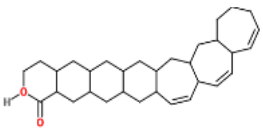
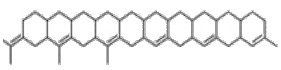


FIGURE 2. GC-MS chromatogram of *C. vespertilionis* leaf extract, peak versus retention time. a) green leaf extract b) red leaf extract (Arrow refers to the anti-inflammatory compound)

oil extract. It also showed chemo-preventive, antioxidant, and antitumor properties (Kelly 1999; Kim & Karadeniz 2012; Rao, Newmark & Reddy 1998; Singab et al. 2015; Singariya, Mourya & Kumar 2015). A compound of 9, 12, 15-octadecatrienoic acid (Z, Z, Z)- and phytol have anti-cancer activity, as reported by Gavamukulya et al. (2015) Sermakkani and Thangapandian (2012), and Suman, Chakkaravarthi Elangomathavan (2013) while n-hexadecanoic acid possessed antibacterial and antioxidant activities (Gavamukulya et al. 2015; Johannes, Litaay & Syahribulan 2016). Anti-inflammatory compounds were discovered in *C. vespertilionis* leaf extract, mainly in a green leaf, using GC-MS analysis, potentially becoming a great therapeutic agent for inflammation.

TABLE 5. Anti-inflammatory compounds in green and red *C. vespertilionis* leaf extract

No	Name of compound/ chemical classes	Retention time (min)	Peak (%)		Molecular formula	Molecular weight	Molecular structure	Biological properties
			Green	Red				
1	n-hexadecanoic acid	20.291	4.679	8.733	$C_{16}H_{32}O_2$	256		-anti-inflammatory (Aparna et al. 2012) -antibacterial (Johannes, Litaay & Syahribulan 2016) -antiandrogenic and antioxidant (Gavamukulya et al. 2015)
2	phytol	21.798	4.715	-	$C_{20}H_{40}O$	296		-antimicrobial and anti-inflammatory (Silva et al. 2014) -anti-cancer, antinociceptive, antioxidant, and antiarthritic (Gavamukulya et al. 2015)
3	9, 12, 15-octadecatrienoic acid, (Z, Z, Z)-	22.092	7.243	1.696	$C_{18}H_{30}O_2$	278		-anti-inflammatory, cancer preventive, and antiarthritic (Sermakkani & Thangapandian 2012; Suman, Chakkaravarthi & Elangomathavan 2013)
4	squalene	27.788	8.814	2.107	$C_{30}H_{50}$	410		-antioxidant, antitumor, and chemopreventive effect (Kelly 1999; Kim & Karadeniz 2012; Rao, Newmark & Reddy 1998; Singab et al. 2015; Singariya, Mourya & Kumar 2015) -anti-inflammatory (Kelly 1999; Lacatusua et al. 2018)

Conclusion

In conclusion, positive biological activities confirm that *C. vespertilionis* leaf extracts, particularly green, contain the most anti-inflammatory compounds. Qualitative analysis showed that this plant contains alkaloids, tannins, saponins, cardenolides, phenolics, cardiac glycosides, flavonoids, and steroids. Total phenolic, flavonoid, and tannin compounds are important secondary metabolite groups responsible for the anti-inflammation activity. The plant showed anti-inflammatory activity when the total phenolic content and protease inhibition activity relationship was similar to the inhibition value of aspirin. Compounds responsible for reducing inflammation were analyzed through GC-MS. These compounds, including n-hexadecanoic acid, phytol, 9, 12, 15-octadecatrienoic acid, (Z, Z, Z)-, and squalene, were present in the green leaf extract, as confirmed by other researchers.

Acknowledgements

The authors are grateful to the University of Malaya for the financial support for this research project through the Research University of Faculty Programme Grant Scheme (GPF006B-2018). We also wish to thank the Institute of Biological Sciences and the Infra Analysis Laboratory for access to their laboratories and infrastructure to perform this research.

References

Abd Rahim, E.N.A., Ismail, A., Nor Omar, M., Rahmat, U.N. & Wan Ahmad, W.A.N. 2018. GC-MS analysis of phytochemical compounds in *Syzygium polyanthum* leaves extracted using ultrasound-assisted method. *Pharmacognosy Journal* 10: 110-119.

Amazu, L.U., Azikiwe, C.C.A., Njoku, C.J., Osuala, F.N., Nwosu, P.J.C., Ajugwo, A.O. & Enye, J.C. 2010. Anti-inflammatory activity of the methanolic extract of the seeds of *Carica papaya* in experimental animals. *Asian Pacific Journal of Tropical Medicine* 3: 884-886.

Aparna, V., Dileep, K.V., Mandal, P.K., Karthe, P., Sadasivan, C. & Haridas, M. 2012. Antiinflammatory property of n-hexadecanoic acid: Structural evidence and kinetic assessment. *Chemical Biology & Drug Design* 80: 434-439.

Apu, A.S., Al-Baizyd, A.H., Ara, F., Bhuyan, S.H., Matin, M. & Hossain, M.F. 2012. Phytochemical analysis and bioactivities of *Argemone mexicana* Linn. leaves. *Pharmacology Online* 3: 16-23.

Ariff, M.A.M., Ghani, Z.A., Razak, N.A.A., Osman, M.S. & Jaafar, J. 2018. Supercritical CO_2 extraction of red butterfly wing leaves: Process parametric study towards extraction yield. *MATEC Web of Conferences* 154: 01015.

Aslam, F., Khalil-ur-Rehman, Asghar, M.K. & Sarwar, M.F. 2009. Antibacterial activity of various phytoconstituents of neem. *Pak. Journal of Agricultural Science* 46: 209-213.

Ayoola, P.B. & Adeyeye, A. 2010. Phytochemical and nutrient evaluation of *Carica papaya* (Pawpaw) leaves. *International Journal of Research and Reviews in Applied Sciences* 5: 325-328.

de Barcelos, I.P., Troxell, R.M. & Graves, J.S. 2019. Mitochondrial dysfunction and multiple sclerosis. *Biology (Basel)* 8(2): 37.

Burke, A., Smyth, E. & FitzGerald, G.A. 2005. Analgesic antipyretic agents, pharmacotherapy of gout. In *Goodman & Gilman's the Pharmacological Basis of Therapeutics*, edited by Brunton, L.B., Lazo, J.S. & Parker, K.L. New York: McGraw-Hill. pp. 671-715.

Canini, A., Alesiani, D., D'Arcangelo, G. & Tagliatesta, P. 2007. Gas chromatography-mass spectrometry analysis of phenolic compounds from *Carica papaya* L. leaf. *Journal of Food Composition and Analysis* 20: 584-590.

Cazarolli, L.H., Zanatta, L., Alberton, E.H., Figueiredo, M.S., Folador, P., Damazio, R.G., Pizzolatti, M.G. & Silva, F.R. 2008. Flavonoids: Prospective drug candidates. *Mini-Reviews in Medicinal Chemistry* 8: 1429-1440.

Cotran, R.S., Kumar, V. & Robbins, S.L. 1994. Inflammation and repair. In *Robbins Pathologic Basis of Disease.* 5th ed. Philadelphia: W.B. Saunders. pp. 51-92.

De Groot, A.S. & Scott, D.W. 2007. Immunogenicity of protein therapeutics. *Trends in Immunology* 28: 482-490.

Debnath, T., Park, S.R., Kim, D.H., Jo, J.E. & Lim, B.O. 2013. Antioxidant and anti-inflammatory activity of *Polygonatum sibiricum* rhizome extracts. *Asian Pacific Journal of Tropical Disease* 3(4): 308-313.

Deepak, P., Axelrad, J.E. & Ananthakrishnan, A.N. 2019. The role of the radiologist in determining disease severity in inflammatory bowel diseases. *Gastrointestinal Endoscopy Clinics of North America* 29(3): 447-470.

Dhanani, T., Shah, S., Gajbhiye, N.A. & Kumar, S. 2017. Effect of extraction methods on yield, phytochemical constituents and antioxidant activity of *Withania somnifera*. *Arabian Journal of Chemistry* 10: S1193-S1199.

Garnock-Jones, P.J. 1983. Note on some folklore drugs and remedies of the Lau Group, Fiji. *South Pacific Journal of Natural Science* 4: 4-8.

Gavamukulya, Y., Abou-Elella, F., Wamunyokoli, F. & El-Shemy, H.A. 2015. GC-MS analysis of bioactive phytochemicals present in ethanolic extracts of leaves of *Annona muricata*: A further evidence for its medicinal diversity. *Pharmacognosy Journal* 7: 300-304.

Gunathilake, K.D.P.P., Ranaweera, K.K.D.S. & Rupasinghe, H.P.V. 2018. Influence of boiling, steaming and frying of selected leafy vegetables on the *in vitro* anti-inflammation associated biological activities. *Plants* 7: 22.

Habila, J.D., Bello, I.A., Dzikwe, A.A., Ladan, Z. & Sabiu, M. 2011. Comparative evaluation of phytochemicals, antioxidant and antimicrobial activity of four medicinal plants native to Northern Nigeria. *Australian Journal of Basic and Applied Sciences* 5: 537.

Hofer, D., Schwach, G., Tabrizi-Wizsy, N.G., Sadjak, A., Sturm, S., Stuppner, H. & Pfragner, R. 2013. *Christia vespertilionis* plant extracts as novel antiproliferative agent against human neuroendocrine tumor cells. *Oncology Reports* 6: 2219-2226.

Johannes, E., Litaay, M. & Syahribulan. 2016. The bioactivity of hexadecanoic acid compound isolated from hydroid *Aglaophenia cupressina lamoureoux* as antibacterial agent against *Salmonella typhi*. *International Journal of Biological and Medical Research* 7: 5469-5472.

Kelly, G.S. 1999. Squalene and its potential clinical uses. *Alternative Medicine Review* 4: 29-36.

Kim, S.K. & Karadeniz, F. 2012. Biological importance and applications of squalene and squalene. *Inorganic Chemistry* 65: 223-233.

Lacatusua, I., Arseniea, L.V., Badeaa, G., Popab, O., Opreaa, O. & Badeaa, N. 2018. New cosmetic formulations with broad photoprotective and antioxidative activities designed by amaranth and pumpkin seed oils nanocarriers. *Industrial Crops and Products* 123: 424-433.

Lee, J.J., Yazan, L.S., Kassim, N.K., Che Abdullah, C.A., Esa, N., Lim, P.C. & Tan, D.C. 2020. Cytotoxic activity of *C. vespertilionis* root and leaf extracts and fractions against breast cancer cell lines. *Molecules* 25(11): 2610.

Lin, J., Opoku, A.R., Geheeb-Keller, M., Hutchings, A.D., Terblanche, S.E., Jager, A.K. & van Staden, J. 1999. Preliminary screening of some traditional Zulu medicinal plants for anti-inflammatory and antimicrobial activities. *Journal of Ethnopharmacology* 68: 267-274.

Mesfin, H. & Won, H.K. 2019. Antioxidant activity, total polyphenol, flavonoid and tannin contents of fermented green coffee beans with selected yeasts. *Fermentation* 5(1): 29.

Mohd Yasin, Z.N., Zakaria, M.A., Nik Mat Zin, N.N.I., Ibrahim, N., Mohamad, F.S., Mat Desa, W.N.S., Sul'ain, M.D. & Abu Bakar, N. 2020. Biological activities and GCMS analysis of the methanolic extract of *Christia vespertilionis* (L.F.) Bakh. F. leaves. *Asian Journal of Medicine and Biomedicine* 4(1): 1-11.

Mokhtar, S.U., Hooi, H.S., Lene, D.T.T. & Jayaraman, S. 2019. Comparison of total phenolic and flavonoids contents in Malaysian propolis extract with two different extraction solvents. *International Journal of Engineering Technology and Sciences* 6(2): 1-11.

Murugesu, S., Perumal, V., Balan, T., Fatinanthan, S., Khatib, A., Arifin, N.J., Mohd Shukri, N.S.S., Saleh, M.S.M. & Hin, L.W. 2020. The investigation of antioxidant and antidiabetic activities of *Christia vespertilionis* leaves extracts. *South African Journal of Botany* 133: 227-235.

Mutalib, N.A. & Latip, N.A. 2019. Synergistic interactions between *Christia vespertilionis* leaves extract and chemotherapy drug cyclophosphamide on WRL-68 Cell Line. *Asian Journal of Pharmaceutical Research and Development* 7(3): 109-113.

NIST. 2011. *Mass Spectral Library, Standard Reference Database 1, NIST 11*. Standard Reference Data Program, National Institute of Standards and Technology. Gaithersburg, MD, USA. https://www.nist.gov/srd/nist-standard-reference-database-1a.

Nguyen-Pouplin, J., Tran, H., Phan, T.A., Dolecek, C., Farrar, J., Tran, T.H., Caron, P., Bodo, B. & Grellier, P. 2007. Antimalarial and cytotoxic activities of ethnopharmacologically selected medicinal plants from South Vietnam. *Journal of Ethnopharmacology* 109: 417-427.

Osman, M.S., Ghani, Z.A., Ismail, N.F., Razak, N.A., Jaapar, J. & Ariff, M.A. 2017. Qualitative comparison of active compounds between red and green *Mariposa Christia Vespertillionis* leaves extracts. In *AIP Conference Proceedings* 1885: 020282.

Oyedapo, O.O. 2001. Biological activity of *Plyllanthus amarus* extracts on Pragrow-Dawley rats. *Nigerian Journal of Biochemistry and Molecular Biology.* pp. 83-86.

Rajurkar, N.S. & Gaikwad, K. 2012. Evaluation of phytochemicals, antioxidant activity and elemental content of *Adiantum capillus veneris* leaves. *Journal of Chemical and Pharmaceutical Research* 4: 365-374.

Rao, C.V., Newmark, H.L. & Reddy, B.S. 1998. Chemopreventive effect of squalene on colon cancer. *Carcinogenesis* 19: 287-290.

Rayburn, E.R., Ezell, S.J. & Zhang, R. 2009. Anti-inflammatory agents for cancer therapy. *Molecular and Cellular Pharmacology* 1: 29-43.

Ruiz-Ruiz, J.C., Matus-Basto, A.J., Acereto-Escoffié, P. & Segura-Campos, M.R. 2017. Antioxidant and anti-inflammatory activities of phenolic compounds isolated from *Melipona beecheii* honey. *Food and Agricultural Immunology* 28: 1424-1437.

Sadava, D., Hillis, D.M., Heller, H.C. & Berenbaum, M.R. 2011. *Life: The Science of Biology.* 9th ed. San Francisco: W.H. Freeman. pp. 105-114.

Sakat, S.S., Juvekar, A.R. & Gambhire, M.N. 2010. *Invitro* antioxidant and anti-inflammatory activity of methanol extract of *Oxalis corniculata* Linn. *International Journal of Pharmacy and Pharmaceuticals Sciences* 2: 146-155.

Sermakkani, M. & Thangapandian, V. 2012. GC-MS analysis of *Cassia italica* leaf methanol extract. *Asian Journal of Pharmaceutical and Clinical Research* 5: 90-94.

Sidek, M.M., Ismail, N.Z., Zain, N.N.M. & Arsad, H. 2019. Cytotoxicity activity of *Christia vespertilionis* leaves extracts against Hela cells and the detection of compounds by GC-MS. *Journal of Biological & Scientific Opinion* 7(6): 71-78. DOI 10.7897/2321-6328.076116

Silva, R.O., Sousa, F.B.M., Damasceno, S.R., Carvalho, N.S., Silva, V.G. & Oliveira, F.R.M.A., Sousa, D.P., Aragao, K.S., Barbosa, A.L.R., Freitas, R.M. & Medeiro, J.V.R. 2014. Phytol, a diterpene alcohol, inhibits the inflammatory response by reducing cytokine production and oxidative stress. *Fundamental and Clinical Pharmacology* 28(4): 455-464.

Singab, A.N., El-Taher, E.M.M., Elgindi, M.R. & Kassem, M.E-S. 2015. *Phoenix roebelenii* O'Brien DNA profiling, bioactive constituents, antioxidant and hepatoprotective activities. *Asian Pacific Journal of Tropical Disease* 5: 552-558.

Singariya, P., Mourya, K.K. & Kumar, P. 2015. Gas chromatography-mass spectrometric analysis of acetone extract of *Cenchrus ciliaris* (Dhaman grass). *International Journal of Science and Nature* 6(4): 652-661.

Suman, T., Chakkaravarthi, K. & Elangomathavan, R. 2013. Phyto-chemical profiling of *Cleistanthus collinus* leaf extracts using GC-MS analysis. *Research Journal of Pharmacy and Technology* 6: 1173-1177.

Shahidi, F. & Yeo, J. 2018. Bioactivities of phenolics by focusing on suppression of chronic diseases: A review. *International Journal of Molecular Sciences* 19(6): 1573.

Sharma, A. & Cannoo, D.S. 2016. Effect of extraction solvents/ techniques on polyphenolic contents and antioxidant potential of the aerial parts of *Nepeta leucophylla* and the analysis of their phytoconstituents using RP-HPLC-DAD and GC-MS. *RSC Adv*ances 6: 78151-78160.

Smitha, S. & Reshma, J. 2019. Anatomical profiling and phytochemical analysis of *Christia vespertilionis* (L.f.) Bakh. f. *International Journal of Pharma and Biological Sciences* 9(Special Issue 1- NCBTIL): 40-50.

Tsai, D.H., Riediker, M., Berchet, A., Paccaud, F., Waeber, G., Vollenweider, P. & Bochud, M. 2019. Effects of short and long-term exposures to particulate matter on inflammatory marker levels in the general population. *Environmental Science and Pollution Research* 26: 19697-19704.

Upadhyay, H.C., Sisodia, B.S., Cheema, H.S., Agrawalb, J., Pal, A., Darokar, M.P. & Srivastava, S.K. 2013. Novel antiplasmodial agents from *Christia vespertilionis*. *Natural Product Communications* 8(11): 1591-1594.

Whiting, P.A. 2007. Commercial production of *Christia subcordata* Moench by establishing cultural practices and by applying plant growth regulators. Master's thesis, The University of Georgia, United States.

*Corresponding author; email: nurairina@um.edu.my