

Antioxidant and antibacterial activity of *Acorus calamus*. L leaf and rhizome extracts¹

Antioksidan dan aktivitas antibakterial ekstrak daun dan rizoma Acorus calamus. L

Kho See Li², Chan Sook Wah²

² School of Biosciences, Taylor's University, Selangor, Malaysia

ABSTRACT

Background: *Acorus calamus* (sweet flag) is a well-known traditional herb used in India. There were only limited studies done on both hydrophilic and hydrophobic extracts of *A. calamus* and the biological activities of phenolic and flavonoid compounds as well as alpha-asarone in sweet flag. **Objective:** The present study was designed to explore the antioxidant and antibacterial activities of both hydrophilic and hydrophobic of *A. calamus* leaf and rhizome extracts. Lastly, the correlation between the biological activities and total phenolic content (TPC), total flavonoid content (TFC) as well as total alpha-asarone content (TAC) of three different extracts (water, hexane and methanol extracts) was revealed. **Method:** TAC was determined through using HPLC method, while TPC and TFC were determined through spectrophotometric method. DPPH-free radical scavenging assay (DPPH-FRS) and ferric reducing power assay (FRAP) were used to analyse antioxidant activity, whereby disc diffusion method was used to analyse the antibacterial activity. **Results:** Methanol extracts had significantly ($p < 0.05$) highest levels of antioxidant activities, antibacterial activity TPC (4.466%¹; 3.603%²) and TFC (12.819%¹; 7.411%²) content. The significant ($p < 0.05$) highest TAC was observed in hexane extracts (0.762%¹; 2.975%²). TPC ($p < 0.05$; $r = 0.855$) and TFC ($p < 0.05$; $r = 0.855$) correlated to FRAP, while DPPH-FRS solely correlated to TFC ($p < 0.05$; $r = 0.873$). **Conclusion:** Both of the hydrophilic and hydrophobic extracts of *A. calamus* could be potent antioxidant agents for nutraceutical application and antibacterial agent for food application. Lastly, flavonoids and phenols extracted from sweet flag could be a good potent source of antioxidants for nutraceuticals or food additives as those compounds are correlated to the antioxidant activity.

¹Leaf extract; ²Rhizome extract

KEY WORDS: *A. calamus*; alpha-asarone; antibacterial; antioxidant; flavonoids; nutraceuticals; phenols; sweet flag

ABSTRAK

Latar belakang: *Acorus calamus* (minyak atsiri) adalah ramuan tradisional terkenal yang digunakan di India. Hanya ada sedikit penelitian yang dilakukan pada kedua ekstrak hidrofilik dan hidrofobik dari *A. calamus* dan aktivitas biologis senyawa fenolik dan flavonoid serta alpha-asarone pada minyak atsiri. **Tujuan:** Mengetahui antioksidan dan aktivitas antibakteri hidrofilik dan hidrofobik daun *A. calamus* dan ekstrak rhizome serta korelasi antara aktivitas biologis dan total phenolic content (TPC), total flavonoid content (TFC) dan kandungan total alpha-asarone (TAC) dari tiga ekstrak yang berbeda (air, heksana, dan ekstrak metanol). **Metode:** TAC ditentukan dengan menggunakan metode HPLC sedangkan TPC dan TFC ditentukan melalui metode spektrofotometri. DPPH-free radical scavenging assay (DPPH-FRS) dan ferric reducing power assay (FRAP) digunakan untuk menganalisis aktivitas antioksidan serta metode disc diffusion digunakan untuk menganalisis aktivitas antibakteri. **Hasil:** Ekstrak metanol memiliki aktivitas antioksidan tertinggi secara signifikan ($p < 0,05$), kandungan aktivitas antibakteri TPC (4,466%¹; 3,603%²) dan TFC (12,819%¹; 7,411%²). TAC tertinggi secara signifikan terlihat pada ekstrak heksana (0,762%¹; 2,975%²) ($p < 0,05$). TPC ($p < 0,05$; $r = 0,855$) dan TFC ($p < 0,05$; $r = 0,855$) berkorelasi dengan FRAP sedangkan DPPH-FRS berhubungan dengan TFC ($p < 0,05$; $r = 0,873$). **Simpulan:** Baik hidrofilik maupun hidrofobik dari ekstrak *A. calamus* dapat menjadi agen antioksidan kuat untuk aplikasi nutraceutical dan agen antibakteri untuk aplikasi makanan. Sementara itu, flavonoid dan fenol yang diekstraksi dari minyak atsiri bisa menjadi sumber potensial baik dari antioksidan untuk nutraceuticals atau pun makanan tambahan sebagai senyawa yang berkorelasi dengan aktivitas antioksidan.

¹Ekstrak daun; ²Ekstrak rimpang

KATA KUNCI: *A. calamus*; alpha-asarone; antibakteri; antioksidan; flavonoid; nutraceutical; fenol; minyak atsiri

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Corresponding author: Kho See Li, School of Biosciences, Taylor's University, No. 1, Jalan Taylor's, 47500 Subang Jaya, Selangor, Malaysia, Tel: +603-56295553; Fax: +603-56295001; e-mail: sookwah.chan@taylors.edu.my

BACKGROUND

The usage of antibacterial and antioxidant as additives in food are common and necessary during food production due to the global food security and food safety reasons. The undesirable oxidation of lipids or pigments and growth of microorganism in food are always associated with some undesirable changes even causing some harmful issues such as development of food spoilage, food-borne illness and off flavours. All those changes are turning the products to unacceptable or unsafe for human consumption. Hence, the action of adding antioxidants and antibacterial agents from external sources is required in order to increase the shelf-life of products. Butylated derivatives are the most widely use synthetic antioxidants as protective agents in a variety of products. However, they have been restricted from usage due to the potential liver damage and carcinogenic (1). The development of new physiological races of pathogens and increasing resistant strengthen in bacteria resulted existing antibacterial agents are becoming ineffective. Thus, there is an urgent request for developing innovative or alternatives of synthetic antioxidant and antibacterial agents to solve the problems. Owing to the negative perception on synthetic antioxidant or antibacterial food that using botanical antioxidant and antibacterial instead of synthetic become a trend of consumers' choice, even some other plant based extracts had used for commercial purpose on large scale through encapsulation technologies (2). Besides that, the consumption of nutraceuticals (products that contain substances which have physiological properties and purified from food, plant, specific diets, as well as processed foods) is increasing rapidly due to the raising health awareness among public. Nutraceuticals which have antioxidant activity are one of the popular nutraceutical products in the market as they are able to prevent life-threatening diseases such as diabetes, cardiovascular diseases and cancers (3). According to Malaysian Adults Nutrition Survey 2012, fish oil is the most popular food supplement that taken by Malaysian adults due to its antioxidant properties (4). The principle of antioxidant to eliminate the initiation or prevent the progression of life-threatening diseases is based on scavenging the free radicals and other reactive oxygen species such as hydroxyl radical, superoxide

anion radical, hydrogen peroxide, oxygen singlet, hypochlorite, nitric oxide radical, and peroxy nitrite radical. As those highly reactive free radicals which can cause the homeostatic unbalance as well as oxidative damages to biological macromolecules such as nucleic acid, proteins, carbohydrates, and lipids (5).

In order to solve the aforesaid problems and demands, *Acorus calamus* or sweet flag could become possible solution as it has reported to possess antioxidant, antimicrobial properties, is a well-known traditional medicinal plant used for numerous diseases treatments in India (6). Thus, this study worked on the extraction of bioactive compounds in terms of antioxidant and antibacterial compounds from *A. calamus* leaf and rhizome involved the use of simple extraction apparatus which is water bath shaker. *A. calamus* can be found in both temperate and sub temperate zones such as India, China, Indonesia, Japan, Australia, southern Russia, southern Siberia, southern Canada as well as the northern United States. Chemical composition of alcoholic *A. calamus* extract and essential oil had been investigated extensively in nearly 20 years. The findings of previous studies demonstrated that the major chemical constituents of non-aqueous *A. calamus* extract were alpha- and beta-asarone. Those bioactive compounds were also found that responsible to most of the biological activities including antioxidant and antibacterial activities. Other than alpha-asarone and beta-asarone, various hydrocarbon compounds also identified in *A. calamus*. Phenols and flavonoids are group as type of hydrocarbon due to presence of hydroxyl group attached to the hydrocarbon ring on their chemical structure (7). Phenolic and flavonoid compounds are also always reported that have correlated with the antibacterial activity and degenerative diseases such as cancer and cardiovascular diseases (8,9).

However, there were limited studies devoted on the aqueous extract of *A. calamus*, thus a comparative study of hydrophobic and hydrophilic extracts of sweet flag was conducted to evaluate their antioxidant and antibacterial activities. Besides that, most of the previous studies focused on the biological activity of beta-asarone as well as alpha-asarone, the biological properties of phenols and flavonoids have not been studied extensively. Despite phenolic and flavonoids are not the major compound

found in sweet flag, numerous of studies of phenolic and flavonoids isolated from fruit and vegetables have been reported to have physiological benefits and sell in the market as nutraceutical products (10). Hence, there is a potential to study the biological activity of phenols and flavonoid from sweet flag. The co-relationship between pharmacological activities (antibacterial and antioxidant) and phenols as well as flavonoids of sweet flag was evaluated through the present study.

METHODS

Plant material

A. calamus plants were provided by PS Green (KL, Malaysia). The leaves and rhizomes were removed from the plant and washed thoroughly with tap water. A known amount of leaves and rhizomes were dried in an oven at 40°C for 24 hours. After drying, the dried leaves and rhizomes were pulverized in a grinder.

Bacteria strains

All the bacteria used in the present study were obtained from the School of Bioscience, Taylor's University Subang Jaya, Malaysia. All of the selected bacteria are commonly found in food including *Bacillus cereus* 10876 (Gram positive), *Escherichia coli* 25922 (Gram negative), *Salmonella. stypthimurium* 13311 (Gram negative) and *Staphylococcus. aureus* 29213 (gram positive).

Reagents and materials

All the reagents and materials used for analyses were tabulated in **Table 1**.

A. calamus leaf and rhizome pulverization

The leaves and rhizomes were removed from the plant and washed thoroughly with tap water. A known amount of leaves and rhizomes were dried in an oven at 40 °C for 24 hours. After drying, the dried leaves and rhizomes were pulverized in a household grinder and the final weight was recorded. The powder of leaf and rhizome was stored separately in Scott bottle at room temperature for further usage.

Extraction and sample preparation

Dried leaf and rhizome powders were dissolved separately in distilled water, methanol and hexane with a ratio 1:20 (g/ml). The extraction was performed at 60°C on a horizontal water bath shaker (EQ-HS-1176 Memmert, Malaysia) with a middle shaking speed condition for 3 hrs. After 3 hours, Whatman No.1 filter papers were used to filter extracts. The solvent was evaporated from the filtrate through a rotary evaporator (Fischer Scientific EYELA, Malaysia) at 40 °C for 10 to 30 mins. For preparing a 200 mg/ml of extract stock solution, a known amount of dried extract was dissolved in DMSO. The mixture was treated with ultra-sonication (Elmasonic S 60H Elma Schmidbauer GmbH, Germany) for 10-15 mins in order to hasten and ensure the full dissolution. Then, membrane filter (0.22 µm) was used to remove the undissolved residues.

Total alpha-asarone content analysis

Total alpha-asarone content of leaf and rhizome extracts was quantified by HPLC (Shimazu Prominence UFLC, Japan) equipped with PDA detector (SPD-M30A Shimadzu, Japan) and C18 column. The injection volume was set as 10 µl of 50 mg/ml of DMSO of extract and a mixture of methanol and distilled water (1:1, v/v) was used as mobile phase to elute the sample from the column at 35 °C (CTO-AS vp Shimadzu, Japan) with a 1.0 ml/min flow rate. The detection of alpha-asarone was carried out at 259 nm at room temperature (11,12). The standard stock solution was prepared by dissolving 10 mg of alpha-asarone in 10 ml of DMSO. A series dilution was performed in order to obtain seven different concentrations of working solution from 0.1 µg/ml to 250 µg/ml.

Total phenolic content analysis

Folin-ciocalteu method was used to determine the total phenolic content of dried leaf and rhizome extracts (13). An amount of 0.1 ml of extract (10mg/ml of DMSO) and 5 ml of diluted folin-ciocalteu were added into a tube. After 3 minutes, 4 ml of 1M of sodium carbonate was added. The assay solution was incubated for 2 hours and measured its absorbance at 765 nm against a blank

Table 1. Reagents and materials used for chemical analyses

Chemical analysis	Chemical name	Purity	Grade
Extraction and sample preparation	Methanol	≥96%	Analytical
	Hexane		Analytical
	Distilled water		
	Whatman No.1 filter paper		
Quantitative of alpha-asarone by HPLC	Dimethyl Sulfoxide(DMSO)	99.9%	Analytical
	Methanol	99.8%	LC
	Distilled water		
	Filter paper		
Total phenolic content (TPC)	C-18		
	Gallic acid	97.5-102.5	
	Anhydrous sodium carbonate	99.9	
	Methanol	≥96%	Analytical
Total flavonoid content (TFC)	Folin-Ciocalteu's reagent		
	Distilled water		
	Catechin	≥98%	HPLC
	Aluminium chloride-6-hydrate	97.0%	
2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity	Sodium nitrite		
	1M sodium hydroxide		
	Distilled water		
	Trolox	98.1%	HPLC
Ferric reducing antioxidant Power (FRAP)	Methanol	≥96%	Analytical
	2,2-diphenyl-1-picrylhydrazyl		
	Gallic acid	97.5-102.5%	
	Methanol	≥96%	Analytical
	Potassium hexacyanoferrate	99.0%	Analytical
	Disodium-hydrogen phosphate monohydrate	99.0%	Analytical
	Sodium-dihydrogen phosphahate	99.0-102%	Analytical
Antibacterial activity	Trichloroacetic acid	99.0%	
	Ferric chloride	99.0%	
	Streptomycin		Analytical standard
	DMSO		
	Mueller-Hinton agar		
	Mueller-Hinton broth		
	Whatman No.1 filter paper		

with DMSO by a using spectrometer (Lambda XLS spectrometer PerkinElmer, UK). The folin-ciocalteu solution (10% v/v) was prepared by transferred a 10 ml of folin-ciocalteu reagent into a 100 ml volumetric flask and diluted it with distilled water. 1M of sodium carbonate was prepared by dissolving 10.5989 g of sodium carbonate in 100 ml of distilled water. A calibration curve was plotted by a series concentration of gallic acid from 200 µg/ml to 1000 µg/ml. The 1000 µg/ml of gallic acid stock solution was prepared by dissolving 25 mg of gallic acid in 25 ml of methanol. The total phenolic contents of extracts were

expressed as gallic acid equivalent (GAE) in grams per 100g of dried extract or percentage.

Total flavonoid content analysis

Quantitative analysis of flavonoid contents of dried leaf and rhizome extracts was determined by using method with slight modification (14). A volume of 0.5 ml of sample with known concentration (10 mg/ml of DMSO) was mixed with 2.5 ml of distilled water and 0.15 ml of 5% (w/v) sodium nitrite. After 6 minutes, 0.3 ml of 10% (w/v) aluminum chloride-6-hydrate and let

the solution stand for 5 mins follow by adding 1.0 ml of 1M sodium hydroxide and 0.5ml of distilled water. The absorbance of the assay was measured at 510 nm against blank with DMSO by using spectrometer (Lambda XLS spectrometer PerkinElmer, UK). The preparation of 5% (w/v) sodium nitrite and 10% (w/v) aluminum chloride-6-hydrate solution were done by dissolving separately of 5 g sodium nitrite and 10 g aluminum chloride-6-hydrate in 100ml of distilled water. Catechin with 1000 µg/ml concentration was used as the standard stock solution by dissolving 25 mg of catechin in 25 ml of distilled water. The calibration curves were plotted with series concentration of gallic acid solution from 2 µg/ml to 400 µg/ml.

2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity

DPPH radical scavenging activities of dried leaf and rhizome extracts were determined by referring the Devi et al. (2011) method (15). A known concentration of 0.1 ml of sample (10mg/ml of DMSO) was mixed with 2mM of DPPH solution. The absorbance of assay was measured at 517 nm after 30 minutes dark incubation by using spectrometer (Lambda XLS spectrometer PerkinElmer, UK). The control tube absorbance was determined by replacing 0.1 ml of sample with 0.1 ml of DMSO. For 2mM DPPH solution preparation was done by dissolving 0.0501 g of DPPH in 100ml of methanol. For calibration curve was plotted with various concentration working solution from 0.2mM to 1mM. The stock solution was prepared by dissolving 0.0501g of trolox in 100ml of methanol. DPPH radical scavenging ability of each sample assay was calculated based on the formula following:

$$\text{Scavenging (\%)} = 1 - \frac{\text{Abs of sample assay}}{\text{Abs of control assay}} \times 100\%$$

Where, Abs=Absorbance

Ferric reducing antioxidant power analysis

Oyaizu method was used to determine the reducing power of *A. calamus* leaf and rhizome extracts with slightly modification (16). The reaction mixture

was prepared by mixing 0.1 ml of plant extracts with 10 mg/ml of DMSO concentration with 2.5 ml of 0.2M phosphate buffer and 2.5 ml of 1% of potassium hexacyanoferrate. Then, the reaction mixture was kept onto water bath (EQ-HS-1176, Memmert, Malayisa) at 50 °C for 20 mins After 20 mins incubation, 2.5 ml of 10% TCA (w/v) was added and filtered through syringe filter (0.45 µm) in order to obtain a clear solution. The filtrate was further diluted with 2.5 ml of distilled water and followed by adding 0.5 ml of 0.05% of ferric chloride. The absorbance was measured at 700 nm against DMSO as blank by spectrometer (Lambda XLS spectrometer PerkinElmer, UK). The preparation of 0.2M of phosphate buffer (pH6.6) was done by mixing approximately 77 ml of 0.2M disodium hydrogen phosphate with 23 ml of 0.2M sodium-hydrogen phosphate. An amount of 2.8392 g disodium hydrogen phosphate and 2.7598 g of sodium dihydrogen phosphate were dissolved separately in 100ml of distilled water. In order to prepare 1% of potassium hexacyanoferrate(w/v), 10% TCA (w/v) and 0.05% of ferric chloride (w/v) solutions, 1 g of potassium hexacyanoferrate, 10g of TCA and 0.05 g of ferric chloride were used to dissolved in 100ml of distilled water respectively. Gallic acid was used as standard and prepared in different concentration from µg/ml to µg/ml.

Antibacterial activity analysis

Disc diffusion method was used to investigate the antibacterial activity of plant extracts through measuring the diameter of formed inhibition zone. A sterile cotton was dipped into cultures broth of bacteria which had approximately 0.2 optical density at 600 nm (17). The sterile cotton was streaked through the surface of sterile Muller Hinton agar plate in order to obtain an even inoculum. After that, a blank loaded with 10 ul of 200 mg/ml of DMSO extract was placed on the agar surface. Simultaneously, blank disc loaded with 10 ul of 1 µg/ml of Streptomycin and 10 ul of DMSO were also placed on agar surface act as positive control and negative control respectively. The agar plate was kept into an incubator at 37 °C for 24 hrs and recorded the diameter of formed inhibition zone.

Statistical analysis

All of the results were reported in mean and standard deviation of triplicate determinations. Independent t-test and Mann-Whitney test were used to determine the significant level between leaf and rhizome extracts obtained using same extracting solvent. While One-way analysis of variance (ANOVA) or Kruskal Wallis test were used for determination of significant level among different solvent extracts of leaf or rhizome. Tukey post hoc test or Tammany were used for further analysis of samples with significant difference at $p < 0.05$. Pearson analysis or Spearman analysis was performed to evaluate the relationship between biological activities and phytochemicals.

RESULTS

Total-alpha-asarone content

Alpha-asarone contents were varied in crude leaf and rhizome extracts obtained from different solvent which fall within 0.01%-3% (Table 2). All the rhizome extracts except for water rhizome extract, showed a significant higher ($p < 0.05$) TAC compared to leaf extracts. Among all the solvents, hexane extracts displayed significant highest TAC among all of extracts obtained from different solvents.

The different small case letters indicate significant difference ($p < 0.05$) in same row. The different digital numbers indicate significant difference ($p < 0.05$) in same column. Values are reported as mean \pm standard deviation of two individual determinations and each determination was performed in triplicate.

Total phenolic content

Total phenolic content of crude leaf and rhizome extracts obtained from different solvents are presented in Figure 1. Methanol leaf extract displayed the significant highest TPC, however, there was no significant different ($p > 0.05$) in terms of TPC in methanol leaf extract and rhizome extract. The significant difference was only found in leaf and rhizome extracts which obtained from water as well as hexane solvents. In leaf extracts, methanol leaf extract showed the significant highest of total phenolic contents followed by water leaf extract and hexane leaf

Table 2. Alpha-asarone contents in different solvents crude extracts of *A. calamus*

	Mean ^a ±SD Alpha-asarone content%	
	Leaf	Rhizome
Methanol	0.27±0.04 ^{a1}	0.46±0.04 ^{b1}
Hexane	0.762±0.126 ^{a2}	2.98±0.11 ^{b2}
Water	0.01±0.00 ^{a3}	0.01±0.00 ^{a3}

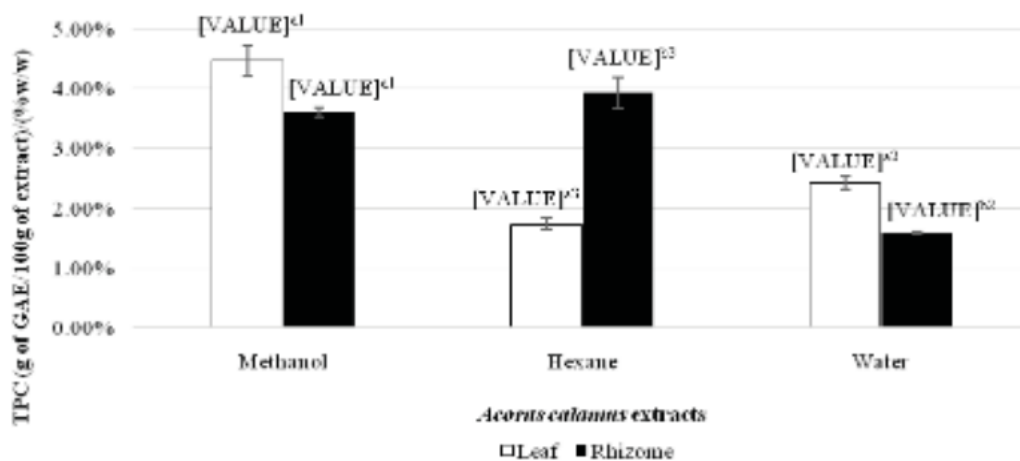


Figure 1. Total phenolic contents of Acorus Calamus leaf and rhizome extracts using 3 different solvents expressed in terms of gallic acid equivalent (g of GAE/100g of extract)/ (w/w%)

extract. While for the rhizome extracts, hexane extracts showed the significant highest ($p < 0.05$) of total phenolic contents followed by methanol and water extracts.

The different small case letters indicate significant difference ($p < 0.05$) between leaf and rhizome of same extracting solvent. The different digital number indicates significant difference ($p < 0.05$) among solvents used to extract same plant part. Values are reported as mean \pm standard deviation of three individual determinations and each determination was performed in triplicate.

Total flavonoid content

According to **Figure 2**, the total flavonoid contents of *A. calamus* extracts varied by the used of solvents and plant parts for extraction. All of the leaf extracts extracted individually from different solvents were identified to contain significant higher amount of flavonoids compared to rhizome extracts ($p < 0.05$). Methanol solvent was found as the most significant effective ($p < 0.05$) to extract flavonoid in both leaf and rhizome followed by water then hexane. It was observed that effect of solvents on TFC is similar to that on TPC in leaf extracts. The highest TFC was methanol extract followed by water extracts and hexane extract.

The different small case letters indicate significant difference ($p < 0.05$) between leaf and rhizome of same

extracting solvent. The different digital number indicate significant difference ($p < 0.05$) among solvents used to extract same plant part. Values are reported as mean \pm standard deviation of three individual determinations and each determination was performed in triplicate.

DPPH-free radical scavenging activity

The antioxidant activity of *A. calamus* could be measured by determine the ability to scavenge free radical. Extract which that display a strong scavenging ability indicated it is a great antioxidant agent. As displayed in **Figure 3**, all the extracts showed different strength of DPPH radical-scavenging. Methanol extract leaf and rhizome extracts showed significant highest level of DPPH scavenging activity. The order of DPPH radical-scavenging activity of the extracts was found to be as follows: methanol rhizome extract, methanol leaf extract, water leaf extract, water rhizome extract, hexane rhizome extract and hexane leaf extract. The ability of DPPH scavenging was found no significant difference ($p > 0.05$) between the leaf and rhizome extracts which obtained from same methanol as well as hexane. However, there was significant difference ($p < 0.05$) in DPPH radical scavenging ability when using different solvents to extract same part of sweet flag, crude extracts obtained from methanol solvent were displayed the

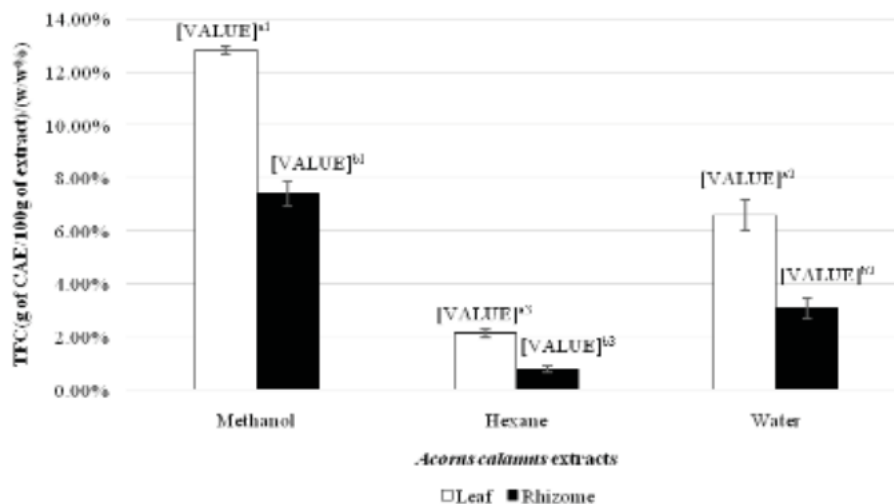


Figure 2. Total flavonoid contents of *Acorus Calamus* leaf and rhizome extracts using 3 different solvents expressed in terms of catechin equivalent (g of CAE/100g of extract)/(w/w%)

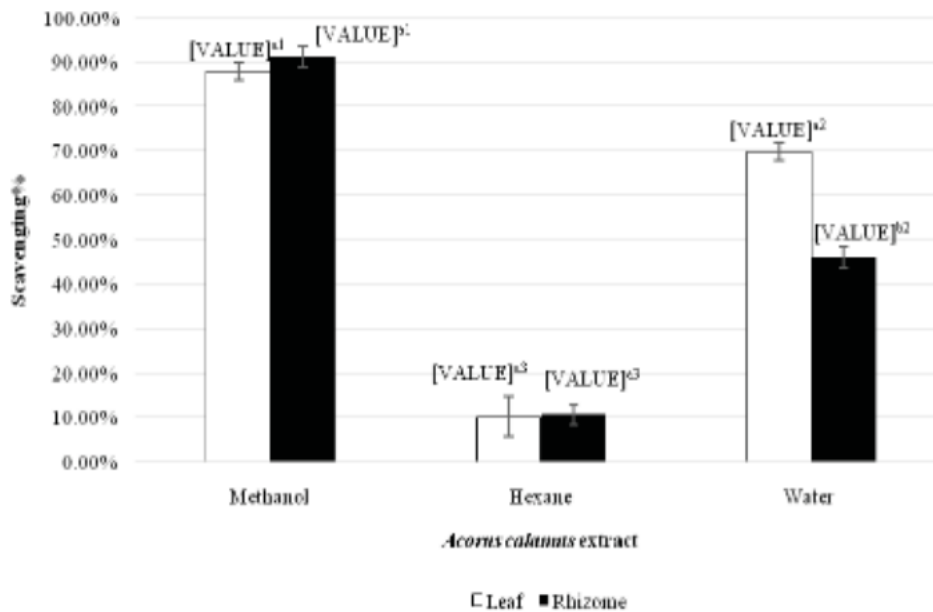


Figure 3. Scavenging percentage of *Acorus Calamus* leaf and rhizome extracts using 3 different solvents

significantly highest DPPH radical scavenging activity among other solvent.

The different small case letters indicate significant difference ($p < 0.05$) between leaf and rhizome of same extracting solvent. The different digital number indicate significant difference ($p < 0.05$) among solvents used to extract same plant part. Values are reported as mean \pm standard deviation of three individual determinations and each determination was performed in triplicate.

Ferric reducing antioxidant power

The antioxidant activity of *A. calamus* could be measured by determine the ability to reduce Fe (III) to Fe(II), as the higher the reducing power indicated the higher the antioxidant activity. All of the extracts shown some degree of reducing ability which presented in Figure 4. The reducing power was various in solvent system. In hexane solvent system, rhizome extracts had significant stronger ($p < 0.05$) reducing powder than leaf extracts, whereas in water system was vice versa. In methanol system, there was no significant different ($p > 0.05$) in term of reducing power. Overall, methanol leaf and methanol rhizome extracts exhibited with highest

reducing power follow by either hexane extracts or water extracts. FRAP was found that correlated to the both TPC and TFC. In addition, TFC ($r = 0.855$) had a stronger co-relationship than TPC ($r = 0.555$) as a greater co-relation coefficient was obtained, but TAC was not correlated to the reducing power.

The different small case letters indicate significant difference ($p < 0.05$) between leaf and rhizome of same extracting solvent. The different digital number indicate significant difference ($p < 0.05$) among solvents used to extract same plant part. Values are reported as mean \pm standard deviation of three individual determinations and each determination was performed in triplicate.

Antibacterial activity analysis

In order to evaluate the antibacterial activity of *A. calamus*, inhibit zone was measured to determine the ability of each extract to inhibit the growth of selected bacteria. The higher the inhibit zone indicated a higher antibacterial activity. The inhibit zone was determined through disc diffusion method and the results are presented in Table 3. All the extracts exhibited comparable antibacterial activity, except for water extracts

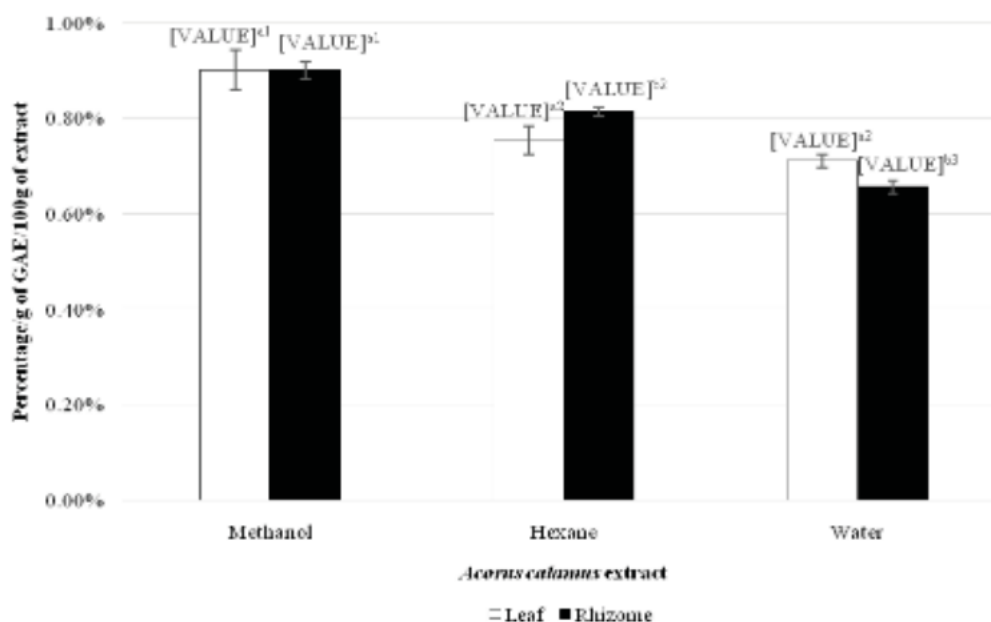


Figure 4. Reducing power of *Acorus Calamus* leaf and rhizome extracts expressed in terms of gallic acid equivalent (g of GAE/100g of extract)

Table 3. Antibacterial activity in different solvents crude leaf and rhizome extract of *A. calamus*

	Inhibition zone(cm)						
	Strep.	MLE	MRE	HLE	HRE	WLE	WRE
<i>B. cereus</i>	1.18±0.15	0.85±0.10	0.9±0.63	0.84±0.19	0.85±0.17	R	R
<i>E. coli</i>	1.77±0.23	0.88±0.10	0.85±0.06	0.90±0.07	0.72±0.05	R	R
<i>S. aureus</i>	1.88±0.11	1.20±0.15	1.20±0.23	0.78±0.05	1.20±0.29	R	R
<i>S. typhi</i>	1.33±0.21	0.78±0.26	1.00±0.00	0.70±0.12	0.77±0.06	R	R
Total	1.57±0.35 ^a	0.93±0.24 ^b	0.99±0.18 ^b	0.80±0.13 ^b	0.89±0.26 ^b	-	-

as no antibacterial activity was observed. Methanol and hexane extracts were demonstrated similar ($p > 0.05$) and substantial antibacterial activity, although both of their antibacterial activities were weaker than the positive control (Streptomycin). In addition, the antibacterial activity was slightly higher in rhizome extracts compared to leaf extracts when using hexane as the extracting solvent, but the difference was not statistically significant. In contrast, the antibacterial activity was slightly higher in leaf extracts compared to rhizome extracts when using methanol as the extracting solvent, but the difference was not statistically significant. TPC and TFC showed weakly positive correlation to antibacterial activity ($p > 0.05$;

$r = 0.175$; $p > 0.05$; $r = 0.237$). However, the antibacterial activity of *A. calamus* extracts did not associate with TPC, TFC and TAC, because of the association was not significantly strong ($p > 0.05$).

The different small case letters indicate significant difference ($p < 0.05$) in the same row. Values are reported as mean \pm standard deviation of two individual determinations and each determination was performed in triplicate. *R=resistant; Strep.= Streptomycin; MLE= methanol leaf extract; MRE= methanol rhizome extracts; HLE= hexane leaf extract; HRE= hexane rhizome extract; WLE= water leaf extract; WRE= water rhizome extract

DISCUSSION

Total alpha-asarone content analysis

Alpha-asarone is one of major compound found in *A. calamus*, according to previous studies, alpha-asarone was also found as the major compound in rhizome oil, which were 9.7% and 5.5%, respectively (2). However, the literature review of alpha-asarone content in leaf was no available (18,19).

The content of alpha-asarone increased as the polarity of extraction solvent decreased. Significant highest ($p < 0.05$) alpha-asarone contents were identified in hexane leaf and rhizome extracts as the hexane is the solvent that possesses the lowest polarity followed by methanol and water. Low polarity behaviour of alpha-asarone molecule could be the cause of this observation. As the functional groups present in alpha-asarone structure (Figure 5) including ether and methyl are least polar and non-polar, respectively. Polarity of ether functional group is approximate to carbon-carbon bond in alkanes, thus ether and methyl functional groups on alpha-asarone structure show a highly affinity to non-polar solvent hexane instead of polar solvents water and methanol (20). Thus, the higher the polarity of solvent, the lower the amount of alpha-asarone being extracted and vice versa.

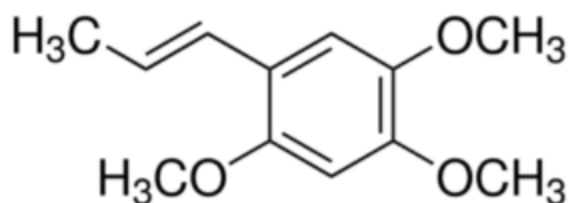


Figure 5. Chemical structure of alpha-asarone (21)

Total phenolic content & total flavonoid content

Hexane rhizome extracts were identified with significant highest TPC among all the rhizome extracts, whereas methanol leaf extracts were identified with the significant highest TPC among all the leaf extracts. A similar finding also obtained, non-polar solvents such as chloroform, benzene, and petroleum ether were found to be more effective to extract phenolic compounds in rhizome than polar solvents such as alcohol and water (22).

It might be a possibility that rhizome of *A. calamus* naturally predominates in complex phenolic compounds

which are less hydrophilic and leaf predominates in more hydrophobic phenolic compound. Generally, high molecular weight or complex phenolic compounds have long carbon side chains, the hydrophilicity of phenols decreased with the increase in the length of hydrophobic carbon side chain which attached on the benzene ring (23). Hence, rhizome of *A. calamus* naturally predominates in complex phenolic compounds might be the possible reason resulted in hydrophobic solvent hexane existed with higher affinity to phenolic compounds in rhizome than hydrophilic solvent water and methanol

Rhizome displayed a significantly higher total phenolic content when using hydrophobic solvent hexane for the rhizome crude extraction. Whereas, leaf displayed a significantly higher total phenolic when hydrophilic solvent methanol was used as extracting solvent. This observation also could be explained with possibility that rhizome of *A. calamus* naturally predominates in complex phenolic compounds (more hydrophobic phenolic compounds), therefore hydrophobic solvent hexane was more suitable to extract phenolic compound in rhizome while hydrophilic methanol was more suitable to extract phenolic compounds in leaf. Lastly, despite both methanol and water are hydrophilic solvent and able to extract phenolic compound, methanol displayed significantly higher phenolic extracting capability than water. It was because of the most of the phenolic compound are partially dissolved in water (24). As the solubility of phenols in water decreases with increase in side chains which attach on benzene ring, thus the non-polar group becomes predominant and masks the effect of polar -OH group to form the hydrogen bond with water. Methanol is an amphiphilic solvent which exhibit both polar and non-polar function group, therefore it shown higher TPC extracting capability than water.

Similar to phenolic compounds, the efficiency of flavonoids extraction depends on the polarity. This also might be due to the flavonoids present in *A. calamus* only partially miscible in water as sweet flag might dominant in high molecular weight of flavonoid with long length of side chain attached on the benzene ring of flavonoid molecule resulted in a low water soluble characteristic. Generally, a mixture of alcohol and water is used to extract flavonoid in plant due to the medium

polar properties of flavonoids (25). Hence, methanol (hydrophilic) was found as the best solvent to extract flavonoids of sweet flag instead of water (extremely hydrophilic) and hexane (non-polar solvent) was found to be worst solvent. In other hands, the leaf of sweet flag might naturally richer in flavonoids than rhizome, thus the higher TFC was observed in leaf extracts.

DPPH free radical scavenging activity

Data obtained from TFC analysis which explained various radical scavenging ability conditions. Strong positive correlation ($p < 0.05$; $r = 0.873$) was found between TFC and scavenging ability. It means that flavonoids might responsible to the free radical scavenging activity of *A. calamus* extracts, the higher the flavonoid content, the stronger the scavenging ability. Despite only TFC strongly associated with DPPH radical scavenging activity, TPC was found weakly correlated ($P > 0.05$; $r = 0.397$) to DPPH free radical.

DPPH assay method is a stable and easy method to determine the potential antioxidant activity level in terms of free radicals scavenging of a substance (26). Thus, sweet flag extracts are suggested as the potential source of antioxidant as all the extracts have showed various free radical scavenging ability. The radical scavenging mechanisms of flavonoids have been investigated through previous structure-activity relationship studies of flavonoids. Figure 6 summarizes the structural criteria that modulate the free radical scavenging activity of flavonoids. The studies have pointed out that the presence of 3',4' dihydroxyl catechol structure in the B ring (Figure 6) possessing electron donating properties and being a radical target. The C2-C3 double bond is conjugated with 4-keto group responsible for electron delocalization from the B ring. Overall, due to the presence of both 3-OH and 5-OH groups in combination with a 4-carbonyl function and C2-C3 double bond, resulting the radical scavenging activity of flavonoids. Hence, flavonoids found in *A. calamus* can be a potent antioxidant as they inhibit the oxidation of radicals by scavenging the radicals through donating electrons to free radicals (27).

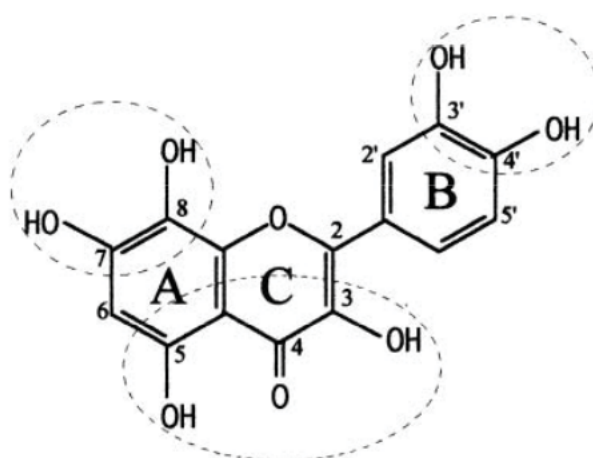


Figure 6. Structure of flavonoids indicating a strong radical scavenging capacity

Similarly, the presence of unsaturated double bond on alpha-asarone structure might be responsible to the radical scavenging properties through electron donation. Study devoted on the antioxidant activity of synthetic alpha-asarone published by Devi et al. (2014), the reduction of DPPH radical in assay caused by an increase of concentration of alpha-asarone (28). This might be due to alpha-asarone and phenols only show their antioxidant properties at a significant concentration. As the amount of alpha-asarone subjected to DPPH assay in the present study [$30 \mu\text{g}$ (0.1 ml of 10 mg/ml of extracts*3%)] was lower than previous study [$200 \mu\text{g}/\text{ml}$ (2 ml of $200 \mu\text{g}/\text{ml}$)]. Lastly, the inactive principle of alpha-asarone presented in the extracts might associate to this observation.

Another finding regarding the antioxidant activity of methanolic extracts of sweet flag reported by Devi, et al (2011), TFC and TPC also found that exhibited positive correlation with DPPH radicals scavenging activity (15). However, only TFC showed the tight correlation to the DPPH radical scavenging activity in this present study. There are few possible causes to this observation. Firstly, low accuracy of TPC results might be generated due to presence of interferences such as vitamin C, protein, sugars and fiber might affect the absorbance and reduce the consistency of TPC. An overestimated result might occur in TPC analysis as interference of non-phenolic compounds by reacting with the Folin-Ciocalteu reagent such as proteins and sugars (29,30). Those compounds

are reported to present in *A. calamus* (Table 4.3) (7), but they do not possess significant free radical scavenging ability. An underestimation result of TPC also might influence the correlation analysis as phenolic compounds of plant always bound to other molecules and unable to react with FC.

Ferric reducing power antioxidant activity

Since TPC and TFC were associated with reducing activity which suggested that phenols and flavonoids present in *A. calamus* could be potent bioactive compounds and the source of antioxidant. A similar findings reported that TPC was positively correlated with reducing activity (17). Oppositely, Devi et al (2014) reported that synthetic alpha-asarone is responsible for the reducing power as the reducing power increased with the increase in the concentration of alpha-asarone from 20 µg/ml to 100 µg/ml (28). This suggested that the amount of alpha-asarone successfully extracted by simple water shaker extraction method might be too low to display the antioxidant properties, thus a weak correlation was observed ($r=0.380$; $p>0.05$). The highest concentration of alpha-asarone subjected to FRAP analysis in the present study [30 µg (0.1 ml of 10 mg/ml of extracts*3%)] was lower than the highest alpha-asarone concentration subjected to FRAP analysis in Devi et al. (2014) [200 µg (2 ml of 200 µg/ml)]. Therefore, this could be possible reason that explained alpha-asarone was not associated with the antioxidant activity in the present study.

Antibacterial activity

According to the correlation results, antibacterial activity of *A. calamus* was not concentration-dependant on TPC, TFC and TAC, hence other bioactive compounds present in the extracts were suspected and contributed to the antibacterial activity. Based on the previous studies, *A. calamus* extracts were reported that existed antibacterial activity (17). Another similar results published by Joshi et al (2012), both the rhizome essential oil and beta-asarone extracted from sweet flag exhibited antibacterial activity against four pathogenic bacteria (31). Therefore, beta-asarone was suspected as another active compound responsible for the antibacterial activity in the present

study. Besides that, beta-asarone also displayed relatively stronger antimicrobial activity than alpha-asarone in the study of Joshi et al (2012). This observation supported the explanation of beta-asarone was suspected as the major compound that contribute antibacterial activity of *A. calamus* extracts instead of alpha-asarone.

Oppositely, an earlier study reported that *Salmonella paratyphi* and *Shigella sonnei*, *Enterococcus faecalis* were resistant to both rhizome and leaf extracts (100 mg/ml of DMSO) except for *Escherichia coli* (17). In addition, weak antibacterial activity of *A. calamus* extract also reported by Phongpaichit, et al (2005) (32). While in this present investigation, all the bacteria were treated with a higher concentration of *A. calamus* extracts (200 mg/ml of DMSO). Hence, positive antibacterial activity results were observed in this preliminary antibacterial activity analysis. Another important finding reported by Devi, et al (2009), pure form of alpha-asarone and beta-asarone existed significantly greater antimicrobial activity than *A. calamus* crude extracts (17). Therefore, non-purified *A. calamus* extracts may also become the possible reason that resulted TAC (alpha-asarone) was not associated with the antibacterial activity in the present study.

Theoretically, the antibacterial activity of *A. calamus* should be strongly correlated with the presence of phenolic and flavonoids. As phenolic acids such as gallic acid and caffeic from other plants have been reported to possess antibacterial activity (33). These compounds were reported present in *A. calamus* in previous study (34). TPC and TFC exhibited weak relationship ($r<0.250$; $p>0.05$) with antibacterial activity of *A. calamus* extracts in this finding. A low bioavailability and low permeability of phenols and flavonoids extracted from *A. calamus* could be the possible reason.

Since purification of *A. calamus* extracts was not conducted in the present study, therefore polyphenols in the *A. calamus* extracts might naturally bound with other compounds such as esters, glycosides and polymers, thus resulting in low permeability against membranes by passive diffusion (35). A low permeability of polyphenols in *A. calamus* might responsible to the weak association between antibacterial activity and TPC as well as TFC. Besides that, natural form of polyphenols usually requires to undergoes biotransformation in term of changing of

structure in gastrointestinal cell and liver cell in order to be absorbed and express their pharmacological activity (35). Therefore, bacterial cell also might require to undergo transformation in order to be metabolized by bacterial cells and exert their antibacterial activity in bacterial cells. According to the TPC and TFC results, the concentration of phenolic and flavonoids is relatively low which is approximately less than 10% except for methanol extracts. A low concentration of bioactive compounds might result in low antibacterial activity. For instance, the phenolic compound will only tend to affect the function of enzyme at low concentration, while they will tend to denature the protein at high concentration (36). Lastly, *A. calamus* rhizome and leaves extracts must contain active principle of derived compound which is believed to be responsible for their antibacterial activity.

CONCLUSION AND RECOMMENDATION

In conclusion, methanol extracts displayed the significant highest antioxidant activity among all of the extracts. Besides that, the highest antibacterial activity also observed in methanol extracts. However, there were no significant differences between methanol leaf extracts and methanol rhizome extracts in term of their antioxidant and antibacterial activity. TPC and TFC correlated with reducing power, while TFC correlated with DPPH radicals scavenging activity which suggested that the phenols and flavonoids are the potent bioactive active responsible to the antioxidant activity. The reason that caused the TPC did not show positive correlation to DPPH free radical scavenging activity might be due to the presence of inference in crude extracts as all of the extracts was not subjected to purification. Despite the antioxidant activity was positively regulated by the concentration of phenols and flavonoids concentration, the antibacterial activity was not. Hence, low bioavailability characteristic of alpha-asarone, flavonoid and phenols might be the reason and only active principle of those compounds would show its biological activity. Lower concentration of alpha-asarone was subjected to antioxidant assay also could be possible reason lead to the observation of antibacterial activity did not correlated to TAC. Besides that, beta-asarone was suspected as major compound

that contributed to antibacterial activity instead of alpha-sarone, flavonoids, and phenols.

Using a more advanced extraction method and conducting purification are recommended for further studies in order to maximize the extraction and avoid the miss conclusion on correlation. Overall, both of the hydrophilic (water and methanol) and hydrophobic extracts (hexane extracts) of *A. calamus* could be the potent antioxidant agent for nutraceutical application and antibacterial agent for food application, except for water extracts as no antibacterial activities were examined. However, a sustainable level of antioxidant activities and amount of phenolic and flavonoid compounds were able to be observed in water extracts. Lastly, flavonoids and phenols from sweet flag extracts could be a good potential source of antioxidant for nutraceuticals or food additives.

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CONFLICTS OF INTERESTS

Authors declare here that do not have any direct financial relation with the plant material supplier mentioned in the paper that might lead to conflict of interests.

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