Science and Technology

RMUTT Journal

Vol.10 No.1 (2020) : 194-206

www.sci.rmutt.ac.th/stj

Online ISSN 2229-1547

Chemicals and Antioxidant Activity of Ethanol Leaf Extract from *Pandanus amaryllifolius* Roxb. Cultivated in Salinity Soil in Ban Donman Village, Maha Sarakham, Thailand

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Abstract

The leaves of *Pandanus amaryllifolius* Roxb. have a strong aroma and are used as flavoring for various food products. They are also widely used in folklore and traditional medicine in rural areas of Thailand. In this research, *P. amaryllifolius* leaves were obtained from local cultivation in Ban Donman village in Maha Sarakham Province, Thailand. 95% ethanol extract of *P. amaryllifolius* leaves (EEPL) was investigated in order to determine its natural potential. Chemical composition of EEPL was analyzed using gas chromatography coupled with mass spectrometry (GC-MS). Twenty six compounds were identified. Elemental analyses of EEPL employed energy dispersive X-ray (EDX) microanalysis, where six elements were identified. The amount of total phenolic compound (TPC) by Folin-Ciocalteu method in the EEPL was 56.46 ± 0.56 mg GAE/g dry weight. The amount of total flavonoid content (TFC) by $AlCl_3$ method was 120.62 ± 7.36 mg QCE/g dry weight. Radical scavenging activity was determined from IC_{50} using DPPH-radical scavenging method. 50% inhibition concentration (IC_{50} values, mg/ml) of standard ascorbic acid was 0.97 ± 0.01 mg/ml, which was more potent than that of EEPL (12.57± 0.45 mg/ml). According to the correlation coefficient, there was a positive correlation between TPC and IC_{50}. Additionally, it was revealed that the solvent in the extraction affected the

Received: May 01, 2020

Revised: May 26, 2020

Accepted: June 19, 2020

amount of TPC, TFC and IC_{50}. This study demonstrates that the 95% ethanol extract of *P. amaryllifolius* leaves cultivated in salinity soil contains compounds with biological activity that can be a good antioxidant source.

Keywords: *Pandanus amaryllifolius* Roxb., chemical composition, total phenol compounds**,** antioxidant activity

1. Introduction

Most herbs are various sources of natural products used in pharmaceuticals, fragrance ingredients, and nutritions. There are well known as one of the highest sources of phytochemicals, which are secondary metabolites generally used importantly in Asia. Each plant synthesizes different individual phytochemicals. Numerous compounds are manufactured by an amazingly diverse network of metabolic pathways. Phenolic and flavonoid compounds are an essential group of secondary metabolites and are also a sort of natural product with antioxidant activities efficient of scavenging free radicals [1-3].

Pandanus amaryllifolius Roxb. or pandan (Pandanaceae). Its Thai name is Toei-hom. The leaves of this plant are used in Southeast Asian cooking for food flavoring as well as in certain applications with medicinal perfume [4-5]. It is generally evident that the secondary metabolites of *P. amaryllifolius* are associated with human benefits. Pandanus leaves contain phytochemicals e.g. steroids, carbohydrates, phenols, isoflavones, coumestrol, lignans, alkaloids, glycosides, amino acids and vitamins [6].

Considering a sustainable development of the local cultivating field area of Maha Sarakham in the northeastern Thailand, which is lack of water, and salinity are as represented environmental problem. There is usually no rain for an extended period. Attractions, Ban Donman village in Maha Sarakham Province is a Sufficiency Economy Learning Center and salinity area, located campus in Mahasarakham University. *P. amaryllifolius* is cultivated traditionally light watering for better performance but here is salinity area, no sufficient watering, the leaf will become grow slowly and will be strong aroma. Biogeographic variation has been shown in various phytochemicals isolated from *P. amaryllifolius* leaves. However, there are no scientific reports on the phytochemicals and antioxidants of *P. amaryllifolius* leaves in the salinity area and it might be expected that salinity influences chemistry, growth and maturity, and other biological properties. Recently, the bioactivity of the phytochemicals from *P. amaryllifolius* has gained increasing interest, at least in terms of the potential health benefits. Therefore, an in-depth investigation of *P. amaryllifolius* leaves is required to assess its potential to be used in medical sciences, especially secondary metabolites with broad pharmacology.

This study was aimed to evaluate the chemical composition by gas chromatography-mass spectrometry (GC-MS) technique. The present investigation was undertaken to determine their total phenol compounds potential and their antioxidant activities in *P. amaryllifolius* collected from salinity soil. Elemental analysis by energy dispersive X-ray (EDX) was also investigated.

2. Materials and Experiments

2.1 Chemicals and reagents

Gallic acid, sodium nitrite, sodium carbonate, aluminum trichloride, sodium hydroxide and quercetin were obtained from Sigma (St. Louis, MO, U.S.A.). 2,2-diphenyl-1-picrylhydrazyl, ascorbic acid (vitamin C), absolute methanol, absolute ethanol and Folin-Ciocalteu's reagent were purchased from Carlo Erba Reagents (Strada Rivoltana, Spain).

2.2 Collection of leaves and extract preparation

Only the sixth leaf from the top of the shoots of *P. amaryllifolius* (1 leaf/plant) was collected; this is uniquely the first fully developed leaf of the plant. The content of chemical composition and storage takes place in the mature leaf. There has been an increased interest in the potential cultivation of *P. amaryllifolius*. Collections were made in the month of March from a local cultivating area of Ban Donman village, Kantharawichai District, Maha Sarakham Province in Thailand. The *P. amaryllifolius* material was authenticated at the Department of Biology, Mahasarakham University. A voucher specimen was deposited in the herbarium of the Natural Medicinal Mushroom Museum at Mahasarakham University, Thailand for future reference. *P. amaryllifolius* leaves were cleaned, air dried and ground to powder. Ten grams of the powder was extracted in 150 ml of 95% ethanol in a Soxhlet extractor (Buchi model B811, Germany). The extract (EEPL) was evaporated, dried and stored at -80 ^{o}C until used.

2.3 Gas chromatography-mass spectrometry (GC-MS) analysis

Mass spectrometry coupled with chromatographic separations, such as gas chromatography (GC-MS), is normally used for the direct analysis of the compounds of medicinal plants. It is applied for the study of medicinal plants as this technique has proved to be a valuable method for the analysis of non-polar components and volatile essential oils, fatty acids, lipids and alkaloids [7].

The Bruker 450-GC and 320-MS are the most sensitive, robust, and flexible quadrupole GC-MS systems currently available. GC-MS analysis of *P. amaryllifolius* leaves was used a Rtx- 5MS capillary column (30m x 0.25mm, fused silica 0.25 um). During the analysis, the furnace temperature was maintained at 110 ^{o}C for 2 min, followed by programming to 200 ^{o}C with a heating speed of 10 ^{o}C /min. The temperature was maintained at 280 ^{o}C for 20 min. The injector and transfer line temperatures were set at 70 ^{o}C and 250 ^{o}C, respectively. The selected injection mode was split (pulsed) with a

split ratio equal to 1/5 with an injection volume set at 2 µl. Pure helium was used as a carrier gas at a flow rate of 1 ml/min. The mass spectrometer was operated in electron impact mode (EI). The mass spectra were recorded at 70 eV standards in the range of m/z 45-500 [8]. Identification of components was achieved by comparing respective retention indices and respective mass spectra to those reported in the literature and in NIST mass spectral library (2008).

2.4 Energy dispersive X-ray (EDX) analysis

The energy dispersive X-ray (EDX) microanalysis is a technique of elemental test related to electron microscopy based on the genesis of characteristic X rays that report the presence of elements present in the sampling [9].

The technique is based on determining the elemental composition using an energy dispersive X-ray (EDX) detector (LEO series 1450 VP, English). The fresh leaf veins of *P. amaryllifolius* on the middle vein line were cut and soaked. All samples were cleaned, fixed with solution and incubated for drying in a hot-air oven for 5 min. The surface of the sample was covered with a very thin layer of carbon by high vacuum evaporation coating or gold using a plasma sputter coater. All samples were detected by the energy and intensity of emitted X-rays used to determine elemental composition. Information on the sampling depth was used to estimate the sampling volume and the number of leaves contributing to the EDX signal for each element [10].

2.5 Qualitative total phenol compounds screening

2.5.1 Total phenolic compounds (TPC) measurement

The Folin-Ciocalteu method is a rapid and widely used to investigate total phenolic compound content [7], [11]. Gallic acid was used as a standard. A hundred microliters of sample was mixed with 2 ml of 2% sodium carbonate and incubated for 2 min. A hundred microliters of Folin-Ciocalteu's reagent (Folin: Methanol, 1:1, v/v) was added, incubated for 30 min and measured at 750 nm. The concentration of total phenolic compounds was determined in milligrams (mg) of gallic acid equivalent (mg GAE/g) using an equation that was obtained from the standard gallic acid plot. TPC was calculated using the following linear regression equation obtained from the standard plot of gallic acid:

$$y = 3.1287x + 0.0037, r^2 = 0.998$$

where y is absorbance and x is the amount of gallic acid in mg.

2.5.2 Total flavonoid content (TFC) measurement

Total flavonoid content was measured by the aluminum chloride colorimetric method [12]. Quercetin standard was dissolved in absolute ethanol. The quercetin solution of 1 mg/ml was used to set up the standard curve. A sample of 200 µl was mixed with 75 µl of 2% sodium nitrite. After incubation at room temperature for 6 min, 150 µl of 10% aluminum chloride was added and incubation was continued for 5 min, after which 500 µl of

sodium hydroxide was added. 1,075 µl of distilled water was used to adjust the net volume. The absorbance of the reaction mixture was measured at 415 nm. The samples were assayed in triplicate and the amount of the flavonoid content was expressed as quercetin equivalent (mg QCE/g). TFC was calculated using the following linear regression equation obtained from the standard plot of quercetin:

$$y = 2.2636x + 0.1773, r^2 = 0.9985$$

where y is absorbance and x is the amount of quercetin in mg.

2.6 Antioxidant activity by DPPH assay

The radical scavenger ability of extract at various concentrations (0.05, 0.1, 0.15, 0.2, and 0.25 mg/ml) was investigated. To 1,950 µl of a methanol solution of 2, 2-diphenyl-1-1 picrylhydrazyl (DPPH), 50 µl of extract solutions were added and the mixture was incubated at room temperature for 45 min. Absorbance was measured at 515 nm against a corresponding blank. The inhibition percentage of DPPH was calculated in the following way: Percentage of inhibition = $[(Abs_{blank} - Abs_{sample})/ Abs_{blank}] \times 100$, where Abs_{blank} is the absorbance of the control reaction (a reaction with all the reagents except the test extract), and Abs_{sample} is the absorbance of the test extract [13]. Tests were carried out in triplicate and the extract concentration providing 50% inhibition (IC_{50}) was obtained by plotting the extract solution concentration versus the inhibition percentage.

2.7 Data analysis

Data from all experiments were analyzed by t-test. The means were compared using the Duncan's Multiple Range Test (DMRT). Values were represented as mean ±SEM of three parallel data sets. *P* values <0.05 were regarded as significant.

3. Results and Discussion

3.1 Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

A total of 26 compounds were identified (Table 1 and Figure 1). Squalene was the dominant component at retention times of 28.311 (17.71%), followed by Phytol 16.770 (14.73%) and 9-Octadecenal 23.051 (14.28%) of the total volatiles, based on peak area data. Other minor constituents included glycerol 1-monooleate 26.417 (8.47%), 9,12-octadecadienal 22.976 (5.12%), docosanoic acid 23.442 (3.59%), linolenic acid 17.730 (1.43%), linoleic acid 17.627 (0.67%), ethyl ester 17.627 (0.67%), and hexadecanoic acid 14.057 (0.66%), which were fatty acids. The presence of five compounds at retention times of gamma-sitosterol 37.127 (6.07%), stigmasterol 35.693 (5.27%), campesterol 34.944 (1.69%) and gamma-tocopherol 31.611 (0.42%), was interpreted to indicate the presence of vitamin E or triterpene.

The compositional analysis for the EEPL indicates the presence of constituents that are known to exhibit medicinal properties as well as physiological activity. GC-MS was able to detect

phytochemical constituents in the extract to some extent. The major components of the EEPL were analyzed by GC chromatogram. Specifically, squalene was the dominant component, accounting for 17.71% of the total volatiles, including phytol, 9-octadecenamide, (Z), gamma-sitosterol, benzofuran, 2, 3-dihydro, gamma-tocopherol and hexadecanoic acid, ethyl ester, which is associated with other phenolic, terpenoids, and alkaloids [14]. Squalene is a natural lipid belonging to the terpenoid family and a precursor of cholesterol biosynthesis. In cosmetics and personal care products, squalene is triterpenoid used in the formulation of a wide variety of products including bath oils, hair products [15]. It is a decomposition product of chlorophyll, but it is not frequently found in essential oils. Various therapeutic activities of phytol have been reported in terms of its activity against myco - bacteria, anticancer activities and antioxidant activity [16-17]. Phytol inhibits the inflammatory response by reducing cytokine production and oxidative stress. Several fatty acid derived compounds, such as, glycerol 1-monooleate, 9, 12-octadecadienal, docosanoic acid, linolenic acid, linoleic acid, ethyl ester and hexadecanoic acid, which were a good source of fatty acid. Saturated and unsaturated volatiles and alcohols are important contributors to the characteristic of flavors in several fruits, vegetables, and also green leaves [18]. These compounds can be formed by the act of thermal of enzymatic lipid oxidation for example, 2-hexenal can be formed by the act of lipoxygenases and also hydroperoxide lyase to the fatty acid substrate, for example, linolenic acid. linoleic acid, an omega-3 fatty acid; and linolenic acid, an omega-6 fatty acid is apparently a major cardioprotective nutrient [19], [2], [20-21]. The human body cannot synthesize essential fatty acids, yet they are critical to human health.

3.2 Energy dispersive X-ray (EDX) analysis

Quantitative EDS X-ray microanalysis using Scanning Electron Microscope (SEM), where six elements were identified (Table 2). The spectrum of the leaf veins of *P. amaryllifolius* on the middle vein line were carbon (C; 33.88 wt.%) shows significant presence of oxygen (O; 8.48 wt.%), sodium (Na; 1.39 wt.%), magnesium (Mg; 0.77 wt.%), chloride (Cl; 0.79 wt.%) and potassium (K; 0.75 wt.%). From table 2, elemental analysis shows salt particles that correspond to Na, Cl and K contents indicative of plants having been grown in a high salinity area. Exhibited Na^+ accumulation and antioxidant during salt stress may play an important role in the cellular toxicity of NaCl in *P. amaryllifolius* and protection mechanism against increased radical production [22-23]. Soil salinity is thought to have a positive impact on the content of TPC, TFC, antioxidant activity, and chemical compositions. The elemental distribution might indicate the favorability of the growth environment from its origin and support the structure and function of plants [16], [24].

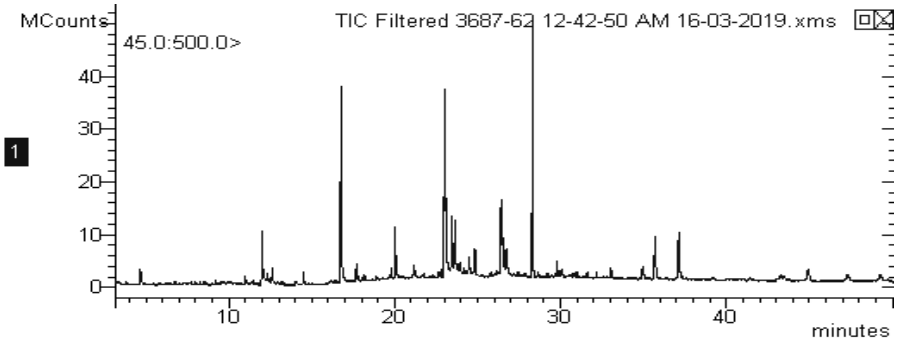

Figure 1 Gas chromatographic separation for 95% ethanol extract of EEPL by GC-MS

Table 1 Biological active chemical compound separation of EEPL by GC-MS

No.	Retention time (min)	Compound Name	Area	%Area	%Prob
1	4.667	Benzofuran, 2,3-dihydro	1.30E±07	1.48	65.2
2	12.006	Phytol acetate	3.44E±07	3.93	45.1
3	12.618	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	1.14E±07	1.30	25.7
4	14.507	Hexadecanoic acid, ethyl ester	5.76E±06	0.66	54.8
5	16.770	Phytol	1.29E+08	14.73	87.6
6	17.627	Linoleic acid, ethyl ester	5.91E±06	0.67	6.9
7	17.730	Linolenic acid, ethyl ester	1.26E±07	1.43	32.4
8	18.188	Octadecanoic acid, ethyl ester	2.59E±06	0.30	30.2
9	19.780	Octanoic acid, 2-dimethylaminoethyl ester	4.42E±06	0.50	17.8
10	20.038	Glycerol 1,3-dipalmitate	3.09E±07	3.52	18.2
11	21.185	9-Octadecenamide, (Z)	7.20E±06	0.82	78.0
12	22.976	9,12-octadecadienal	4.49E±07	5.12	14.6
13	23.051	9-octadecenal	1.25E±08	14.28	19.5
14	23.442	Docosanoic acid	3.15E±07	3.59	18.5
15	24.813	2,6,10,15,19,23-Hexamethyl-tetracosa	2.18E±07	2.49	48.7
16		2,6,10,14,18,22-Hexaene-12,13-diamine			
17	26.417	Glycerol 1-monooleate	7.43E±07	8.47	43.0
18	26.510	alpha-Glyceryl linolenate	2.90E±07	3.31	13.6
19	28.311	Squalene	1.55E±08	17.71	31.1

No.	Retention time (min)	Compound Name	Area	%Area	%Prob
20	29.767	Farnesol isomer a	8.33E±06	0.95	5.6
21	31.611	Gamma-tocopherol	3.72E±06	0.42	54.9
22	32.173	Hentriacontane	4.11E±06	0.47	5.6
23	33.029	Dl-alpha-tocopherol	7.26E±06	0.83	42.6
24	34.944	Campesterol	1.49E±07	1.69	44.2
25	35.693	Stigmasterol	4.62E±07	5.27	43.4
26	37.127	Samma-sitosterol	5.32E±07	6.07	69.5
	Total		8.77E±08	100.00	

Table 2 Elemental composition using an energy dispersive X-ray (EDX) microanalysis

No.	Elemental analysis	Content %
1	Carbon (C)	33.88
2	Oxygen (O)	8.48
3	Sodium (Na)	1.39
4	Magnesium (Mg)	0.77
5	Chloride (Cl)	0.79
6	Potassium (K)	0.75

Table 3 Comparison of total phenolic compound and total flavonoid content

No.	Parameter analyzed	Values obtained	P-value	TFC/TPC
1	Total phenolic compound (mg GAE/g dry weight)**	56.46 ±0.56	0.021*	2.14
2	Total flavonoid content (mg QCE/g dry weight)***	120.62±7.36		

The values are mean ± SEM: (n=3): ** Gallic acid equivalent: ***Quercetin equivalent: * P<0.01.

Table 4. DPPH free radical scavenging activity of EEPL

Sample	Correlation Coefficient			
	EEPL	IC_{50} Values	Total phenolic compound	Total flavonoid content
IC_{50} Values (mg/ml)	12.57±0.45	1	0.19	-1.00**
Ascorbic acid	-	0.97±0.01	-	-
Total phenolic	-	0.19	1	-0.19
compound	-	1.00**	-0.19	1
Total flavonoid	0.018*	-	-	-
content				
P-value				

The values are mean ± SEM: (n=3): * P<0.01. ** Correlation is significant at the 0.01 level (2-tailed).

3.3 Total phenolic compounds (TPC) measurement

EEPL had a TPC of 56.46 ±0.56 mg GAE/g dried weight. In contrast, Jimtaisong and Krisdaphong [25] found higher TPC content than reported here EEPL, but we used the only the sixth leaves of *P. amaryllifolius*. It was reported that the number of other volatiles increased effectively with increase in quantity of plant material, 2AP compound and chemical compounds were significantly less in the young leaves (1–5th leaf from apex), increased and remained almost constant from 6th leaf onwards up to 23rd leaf [14]. It was observed that different plants contained different amounts of TPC. TPC was also different depending on plant types and parts. It appeared that EEPL contained TPC (Table 3).

The quantities of TPC and TFC were compared. The TFC values of EEPL showed a higher amount than TPC at a TFC/TPC ratio of 2.14. The proportions for TFCs/TPCs of EEPL are presented in Table 3. TPC and TFC are very important constituents in plants because of the scavenging ability of hydroxyl groups and the ability to prevent the decomposition of hydroxyl peroxides into free radicals [23].

3.4 Total flavonoid content (TFC) measurement

The total flavonoid content (TFC) of the extract was 120.62 ± 7.36 mg QCE/g dried weight (Table 3). TFC was noticeable that the different solvents in the extraction procedure affected the contents and the subgroups of the extract constituents. This study showed that EEPL significantly contained amounts of TFC higher than

TPC values. Due to *P. amaryllifolius* leaves being sources of natural colorants, which are mostly of carotenoid origin. According, *P. amaryllifolius* contains phytochemicals e.g. steroids, carbohydrates, phenols, isoflavones, coumestrol, lignans, alkaloids, glycosides, amino acids and vitamins. Plants produce diverse chemical with respect to volatile monoterpene and phenylpropene content has been documented [24] in leaves, skin and seeds by detecting TPC and TFC [23].

3.5 Antioxidant activity by DPPH assay

The correlation between the amounts and antioxidant activity of EEPL is expressed as a correlation coefficient, as presented in Table 4. The fifty percent of radical scarvenger (IC_{50}) of EEPL was 12.57 ± 0.45 mg/ml which was higher than 2.34 ± 0.040 mg/ml reported in [25]. The correlation coefficients were 0.19 and 1.00 for IC_{50} and TPC, respectively. The increase of IC_{50} values caused an increase of TPC, meaning that the effectiveness of the radical scavenging activity was a reverse relationship to the amounts of TPC in the extract. Based on the few correlation coefficients (r=0.19), it is possible that there were other secondary metabolites, not only radical scavenging activity. Free radical scavenging activity of polyphenol has been associated with the number and position of hydroxyl groups [22]. However the position of these groups, in a molecule, has greater impact on the exerted activity. Compounds which produce relation stable resulting antioxidant activity. For the amounts of TFC, the correlation coefficient was -0.19, which was an indirect relation. It means that the antioxidant activity of extract was increased along with an increase of TFC.

It is suggested that plants produce several different phytochemicals in their secondary metabolic pool. The results of this study found that the antioxidant activity of the extract from *P. amaryllifolius* leaves was very efficient as a free radical scavenger due to the lowest IC_{50} value. In addition, *P. amaryllifolius* leaves were collected the sixth with a unique and season the month in relation. In conclusion, the extract of *P. amaryllifolius* leaves could be a good selective choice for further study of the source of antioxidants.

4. Conclusions

The sixth leaf (the first leaf of maturity selected from the top of leaf initiation) of *P. amaryllifolius* was found to have a unique high essential oil yield and substances. The results from this study revealed the crude extract from plants, the geographical locations and the environmental condition of Ban Donman village, Kantharawichai District, Maha Sarakham Province in the Northeast of Thailand. The presence of antioxidant activity and GC-MS analysis were taken understand the nature of the active principles in this plant and some phytochemical will be helpful for further detailed study. 95% ethanol extract of *P. amaryllifolius* leaves exhibited the best total flavonoids and showed inverse proportion with the efficacy of total phenolic compound as well as antioxidant activity. The 95% ethanol extract of *P. amaryllifolius* leaves contained compounds with biological activity and

was a good source of fatty acids that can be used to more eat associated with a healthier fatty acid profile and a good source of antioxidant content. Thus, it could be a selective choice for further study involving in-vivo antioxidants and others pharmacological activity.

5. Acknowledgement

This work was financially supported by the Faculty of Science, Maha Sarakham University, Thailand. I am also grateful to Miss Wipapan Khammungkun for her help and assistance throughout the period of this research.

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