XANTHINE OXIDASE INHIBITORY ACTIVITY OF *Pandanus amaryllifolius* Roxb.

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ABSTRACT

Xanthine oxidase (XO) is a pivotal enzyme in purine metabolism. As the end product, overproduction of uric acid may lead to hyperuricemia and gout disease. This research was conducted to assess the antioxidant potential and xanthine oxidase inhibitory activity of *Pandanus amaryllifolius* Roxb. *P. amaryllifolius* was extracted with hexane, petroleum ether, chloroform, methanol and distilled water, where the crude extracts were named as PA-H, PA-PE, PA-C, PA-M, and PA-W respectively. The TLC result showed the presence of terpenoids in all of the extracts. The total phenolic and flavonoid contents were conducted in *P. amaryllifolius* extracts. PA-W showed the highest TPC with 12.88 ± 0.43 mg GAE/g of dry extract whereas PA-PE showed the highest TFC with 15.02 ± 0.58 mg QE/g of dry extract. Antioxidant activities were performed on *P. amaryllifolius* extracts and PA-W generally exhibited the highest activity. In DPPH, metal chelating, and hydrogen peroxide assay, all extracts displayed low scavenging activity. However, each extract possessed steady increase in inhibition activity within their concentration range. PA-W showed the highest activity in DPPH (>240 μg/mL), metal chelating (>160 μg/mL), and hydrogen peroxide assay (>320 μg/mL). PA-M showed the highest activity in FRAP assay (64.39 ± 2.79 mmol Fe^{2+}/g of dry extract). TPC revealed positive significant correlation with DPPH (r = 0.972, $P<0.01$), FRAP (r = 0.964, $P<0.01$), and hydrogen peroxide assay (r = 0.898, $P<0.05$), but no correlation with metal chelating assay (r = 0.382) and *in vitro* XO inhibitory activity (r = 0.809). TFC showed negative correlation with all assays. In *in vitro* XO inhibitory activity, PA-W displayed the highest activity (>100 μg/mL). In *in vivo* XO inhibitory activity, acute toxicity test of PA-W tested at 2 g/kg body weight showed no signs of toxicity and mortality after 14 days. The treatment with PA-W showed significant ($P<0.001$) decrease in serum uric acid level and XO activity. At the dose of

1000 mg/kg body weight and 500 mg/kg body weight, the serum uric acid level were 2.55 ± 2.23 mg/dL and 6.08 ± 1.00 mg/dL, whereas the XO activity were 3.84 ± 0.68 mu/mL and 6.35 ± 0.87 mu/mL, in hyperuricemic rats. Allopurinol standard exhibited serum uric acid level and XO activity of 1.72 ± 1.01 mg/dL and 1.06 ± 0.21 mu/mL respectively. Thus, the results of this finding support the use of *P. amaryllifolius* in reducing uric acid for the treatment of hyperuricemia and rheumatoid arthritis.

ABSTRAK

Xanthine oksidase ialah satu enzim penting dalam metabolism purin. Sebagai produk akhir, penghasilan berlebihan asid urik boleh mengakibatkan penyakit hiperurisemia dan gout. Kajian ini dijalankan untuk menilai potensi antioksidan dan aktiviti perencatan xanthine oksidase *Pandanus amaryllifolius* Roxb. *P. amaryllifolius* telah diekstrak dengan heksana, petroleum eter, klorofom, metanol dan air suling, di mana ekstrak mentah itu dinamakan sebagai PA-H, PA-PE, PA-C, PA-M, dan PA-W masing-masing. Hasil TLC menunjukkan kehadiran terpenoid dalam semua ekstrak. Jumlah kandungan fenolik dan flavonoid telah dijalankan ke atas ekstrak *P. amaryllifolius*. PA-W menunjukkan TPC tertinggi dengan 12.88 ± 0.43 mg GAE/g ekstrak kering manakala PA-PE menunjukkan TFC tertinggi dengan 15.02 ± 0.58 mg QE/g ekstrak kering. Aktiviti antioksidan telah dijalankan ke atas ekstrak *P. amaryllifolius* dan PA-W umumnya menunjukkan aktiviti tertinggi. Dalam asei DPPH, logam pengkelat dan hidrogen peroksida, semua ekstrak memaparkan aktiviti memerangkap yang rendah. Namun, setiap ekstrak menunjukkan peningkatan kukuh dalam aktiviti perencatan dalam julat kepekatan mereka. PA-W menunjukkan aktiviti tertinggi dalam asei DPPH (>240 μg/mL), logam pengkelat (>160 μg/mL), dan hidrogen peroksida (>320 μg/mL). PA-M menunjukkan aktiviti tertinggi dalam asei FRAP (64.39 ± 2.79 mmol Fe^{2+}/g ekstrak kering). TPC menunjukkan kolerasi positif signifikan dengan asei DPPH ($r = 0.972$, $P<0.01$), FRAP ($r = 0.964$, $P<0.01$), dan hidrogen peroksida ($r = 0.898$, $P<0.05$), tetapi tiada kolerasi dengan asei logam pengkelat ($r = 0.382$) dan aktiviti perencatan XO *in vitro* ($r = 0.809$). TFC menunjukkan kolerasi negatif dengan semua asei. Dalam aktiviti perencatan XO *in vitro*, PA-W menunjukkan aktiviti tertinggi (>100 μg/mL). Dalam aktiviti perencatan XO *in vivo*, ujian ketoksikan akut PA-W diuji pada 2 g/kg berat badan tidak menunjukkan tanda-tanda keracunan dan kematian selepas 14

hari. Rawatan dengan PA-W menunjukkan penurunan signifikan (P<0.001) dalam tahap asid urik dan aktiviti XO serum. Pada dos 1000 mg/kg berat badan dan 500 mg/kg berat badan, tahap asid urik serum ialah 2.55 ± 2.23 mg/dL dan 6.08 ± 1.00 mg/dL manakala aktiviti XO ialah 3.84 ± 0.68 mu/mL dan 6.35 ± 0.87 mu/mL, pada tikus hiperurisemik. Piawai allopurinol memaparkan tahap asid urik dan aktiviti XO serum 1.72 ± 1.01 mg/dL dan 1.06 ± 0.21 mu/mL masing-masing. Oleh itu, keputusan daripada penemuan ini menyokong penggunaan *P. amaryllifolius* dalam mengurangkan asid urik untuk rawatan hiperurisemia dan radang sendi.

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LIST OF SYMBOLS AND ABBREVIATIONS

2AP	:	2-acetyl-1-pyrroline
$A_{control}$	:	absorbance of control
$A_{sample\ or\ standard}$	:	absorbance of sample or standard
Allopurinol	:	1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one
AMP	:	adenosine monophosphate
ANOVA	:	analysis of variance
ATP	:	adenosine triphosphate
$C_5H_4N_4O_3$	:	uric acid
cAMP	:	cyclic adenosine monophosphate
CKD	:	chronic kidney disease
cm	:	centimeter
CMC	:	carboxymethyl cellulose
DMSO	:	dimethyl sulfoxide
DNA	:	deoxyribonucleic acid
DPPH	:	2,2-Diphenyl-1-picrylhydrazyl
EDTA-$Na_2.2H_2O$	:	ethylenediaminetetraacetic acid disodium salt dehydrate
EI	:	electron ionization
ESI	:	electrospray ionization
F_{254}	:	fusion protein sequence 254
FAD	:	flavin adenine dinucleotide
$FADH_2$	:	flavin adenine dinucleotide (reduced form)
FDA	:	Food and Drug Administration
Fe/S	:	iron-sulphur
Fe^{2+}	:	ferrous ion
Fe^{3+}	:	ferric ion
$FeCl_2$	:	ferrous chloride

FRAP	:	ferric reducing antioxidant power
g	:	Gram
GAE	:	gallic acid equivalent
GC-MS	:	gas chromatography-mass spectrometry
GMP	:	guanosine monophosphate
GTP	:	guanosine-5'-triphosphate
H_2O_2	:	hydrogen peroxide
H_2SO_4	:	sulphuric acid
HCl	:	hydrogen chloride
HD	:	high dose
HPLC	:	high-performance liquid chromatography
i.p.	:	intraperitoneal injection
IC_{50}	:	half maximal inhibitory activity
IMP	:	inosine monophosphate
kDa	:	kilodalton
kg	:	kilogram
LC-MS	:	liquid chromatography-mass spectrometry
LD	:	low dose
LSD	:	least significant difference
m	:	meter
M	:	molar
mg	:	milligram
mg/dL	:	milligram/deciliter
mg/kg	:	milligram/kilogram
mg/mL	:	milligram/milliliter
mu/mL	:	milliunit/milliliter
min	:	minute
mL	:	milliliter

mL/min	:	milliliter/minute
mM	:	millimolar
mmol	:	millimole
MS	:	mass spectrometer
MSTFA	:	N-methyl-N-(trimethylsilyl)-trifluoroacetamide
MSU	:	monosodium urate
NaCl	:	sodium chloride
NAD^+	:	nicotinamide adenine dinucleotide
NADH	:	nicotinamide adenine dinucleotide (reduced form)
NADPH	:	nicotinamide adenine dinucleotide phosphate
nm	:	nanometer
NSAID	:	nonsteroidal anti-inflammatory drugs
O^-	:	superoxide anion
O_2	:	oxygen
OECD	:	Organisation for Economic Co-operation and Development
PA-C	:	*Pandanus amaryllifolius* chloroform extract
PA-H	:	*Pandanus amaryllifolius* hexane extract
PA-M	:	*Pandanus amaryllifolius* methanol extract
PA-PE	:	*Pandanus amaryllifolius* petroleum ether extract
PA-W	:	*Pandanus amaryllifolius* water extract
pH	:	potential of hydrogen
PO	:	oxonic acid potassium salt or potassium oxonate
ppm	:	parts per million
QE	:	quercetin equivalent
Q-TOF	:	quadruple time-of-flight
r	:	Pearson correlation coefficient
R_f	:	retention factor
RNA	:	ribonucleic acid

ROS	:	reactive oxygen species
RT	:	retention time
SCARs	:	serious cutaneous adverse reactions
SD	:	Sprague Dawley rats
SEM	:	standard error of the mean
TENS	:	toxic epidermal necrolysis syndrome
TLC	:	thin layer chromatography
TLS	:	tumor lysis syndrome
TPTZ	:	2,4,6-Tris(2-pyridyl)-s-triazine
UV	:	ultraviolet
v/v	:	volume/volume
XDH	:	xanthine dehydrogenase
XO	:	xanthine oxidase
XOR	:	xanthine oxidoreductase
μg	:	microgram
μL	:	microliter
μM	:	micromolar
%	:	percentage
<	:	less than
>	:	more than
°C	:	degree Celsius

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

Xanthine oxidase (XO) which is in a form of molybdoflavin protein xanthine oxidoreductase (XOR) serves a crucial role in the degradation of purine in humans (Hille, 2005). The final metabolite in purine catabolism in humans is uric acid (Lima *et al.*, 2015). In the process, hypoxanthine is converted to xanthine and xanthine is converted to uric acid via enzymatic reaction of xanthine oxidase (Mamat *et al.*, 2014). Uric acid generated is then discharged in the urine. Excessive or little manufacture of uric acid could consequently result in hyperuricemia and gout (Richette & Bardin, 2010).

According to Boffetta *et al*. (2009) hyperuricemia is a prominent threat factor for gout, hypertension as well as diabetes. The basis of the disease is uric acid crystallization and deposition in joints and neighboring tissues. Since uric acid overproduction is the key causation of hyperuricemia, the ultimate assuring target for therapeutic remedy of this disorder is xanthine oxidase (Richette & Bardin, 2010). According to Umamaheswari *et al*. (2007) 5 to 30% of worldwide human population experiences uric acid overproduction or underexcretion. Gout which is described as a steady increase in plasma urate levels has a prevalence of about 1% whereas chronic kidney disorder that is usually linked to hyperuricemia has a prevalence of about 5% (Richette & Bardin, 2010).

Treatment of illness related to hyperuricemia has been tackled by the means of keeping the precipitation of plasma urate concentrations low as well as getting rid of the present urate crystals (Kumar & Azmi, 2014). According to Richette & Bardin (2010) at the present time, allopurinol and febuxostat are the available drugs used to obstruct xanthine oxidase activity and reduce serum uric acid levels. The most frequently used

drug for the treatment of chronic gout is allopurinol and it has been used for more than 40 years clinically. Despite that, due to hypersensitivity or intolerance to the drug, or failure of the treatment, allopurinol cannot be used on certain patients. This is because of life-threatening undesirable effects of allopurinol like hypersensitivity syndrome which includes fever, skin rash, eosinophilia, hepatitis, and renal toxicity. These conditions possess mortality rate near to 20%. As stated by Lü *et al*. (2013) we are in need of finding new xanthine oxidase inhibitors with more precise effects and fewer side effects as compared to allopurinol and febuxostat in order to inhibit and fight gout and cardiovascular diseases associated with hyperuricemia.

Natural medicines of plant origin portrayed a very notable role in human disease therapy and impediment for a thousand years globally (Chin *et al*., 2006). Balunas & Kinghorn (2005) stated that natural products revealed from medicinal plants contributed an abundance of clinically helpful medicament and are foreseen to continue being a fundamental role in the new medicines research. *Pandanus amaryllifolius* Roxb. is a common plant found in South East Asia and often known as pandan (Nor *et al*., 2008). In traditional medical field of South East Asia, *P. amaryllifolius* leaves are useful for body revival, fever reducing and also relieving indigestion and flatulence (Cheeptham & Towers, 2002). Apart from that, the leaves of *P. amaryllifolius* are extensively used to enhance flavour in assorted food dishes (Bhattacharjee *et al*., 2005). To the extent of our knowledge, currently no one has examined the effects of *P. amaryllifolius* on *in vitro* and *in vivo* xanthine oxidase inhibitory activity. For this reason, this medicinal plant was selected for study. The outcome of this research will be beneficial to discover novel xanthine oxidase inhibitor derived from natural substances as an effective therapy for hyperuricemia in Malaysia.

1.1 Research objectives

1. To identify the phytochemical compounds in *P. amaryllifolius* using Thin Layer Chromatography (TLC), Gas Chromatography Mass Spectrometry (GC-MS) and Liquid Chromatography Mass Spectrometry (LC-MS).
2. To quantify the phenolic and flavonoid content in *P. amaryllifolius*.
3. To determine the antioxidant activity of *P. amaryllifolius*.
4. To evaluate the xanthine oxidase inhibitory activity of *P. amaryllifolius* in *in vitro* and *in vivo* model.

CHAPTER 2: LITERATURE REVIEW

2.1 Purine metabolism

According to Ishikawa *et al.* (2013) purines are the constituents of nucleosides which are the building blocks of DNA and RNA. The nucleosides of purine such as adenosine and guanine, are typically utilized in order to make other crucial elements in metabolic system like adenosine triphosphate (ATP), guanosine-5'-triphosphate (GTP), cyclic adenosine monophosphate (cAMP), nicotinamide adenine dinucleotide (NADH), and nicotinamide adenine dinucleotide phosphate (NADPH). The chemical structure of purine and its derivatives family such as adenine, guanine, isoguanine, xanthine, hypoxanthine, uric acid, caffeine, and theobromine are depicted in Figure 2.1.

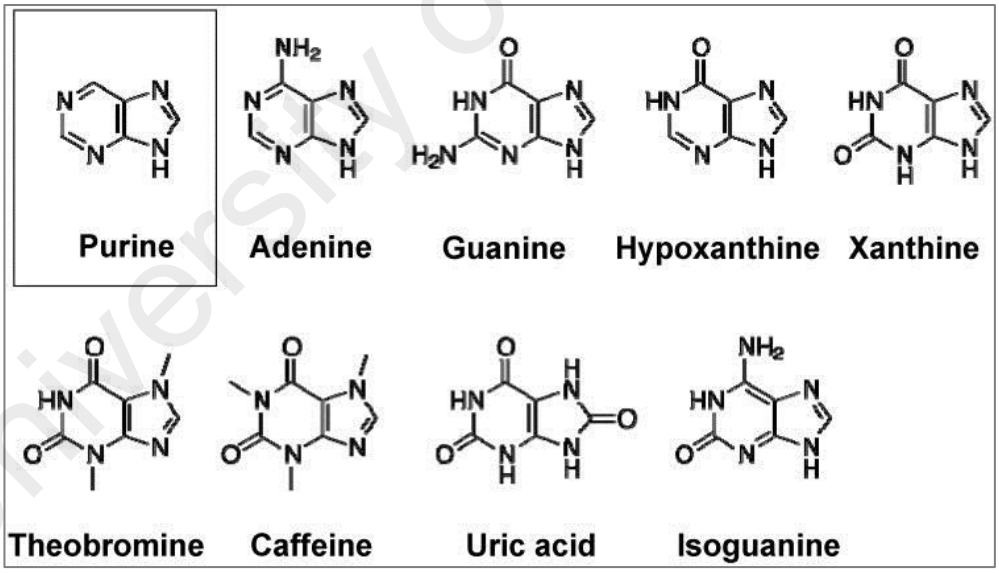

Figure 2.1: Chemical structures of purine and its derivatives, i.e., adenine, guanine, hypoxanthine, xanthine, theobromine, caffeine, uric acid, and isoguanine (Ishikawa *et al.*, 2013)

In the metabolic pathway of purine catabolism, to convert two purine nucleic acids, adenine and guanine, to uric acid, the process needs the presence of multiple enzymes (Jin *et al.,* 2012). The process comprise of various steps of reactions using different enzymes such as AMP deaminase, 5'-nucleotidase, adenosine deaminase, purine nucleoside phosphorylase, guanine deaminase and xanthine oxidase (Ishikawa *et al.,* 2013).

In the beginning, AMP (adenosine monophosphate) is converted to inosine by two separate steps. First, AMP in converted to IMP (inosine monophosphate) by removing an amino group via enzyme AMP deaminase then followed by dephosphorylation to form inosine using enzyme 5'-nucleotidase; or secondly, by converting AMP to adenosine by removing a phosphate group via enzyme 5'-nucleotidase followed by deamination reaction of adenosine to form inosine using enzyme adenosine deaminase.

GMP (guanine monophosphate) undergo conversion to form guanosine by enzyme 5'-nucleotidase. The nucleosides which are the inosine and guanosine, are then converted to purine base hypoxanthine and guanine, respectively by enzymatic reaction of PNP (purine nucleoside phosphorylase). Hypoxanthine undergoes oxidation to become xanthine by enzyme xanthine oxidase, whereas guanine undergoes deamination to become xanthine by enzyme guanine deaminase.

Xanthine is then further converted by oxidation to form uric acid via xanthine oxidase (Maiuolo *et al.*, 2016). Thus, any incident of the abnormalities occur in this pathway increases the level of uric acid (Wong *et al.,* 2014). The purine catabolism pathway is depicted in Figure 2.2.

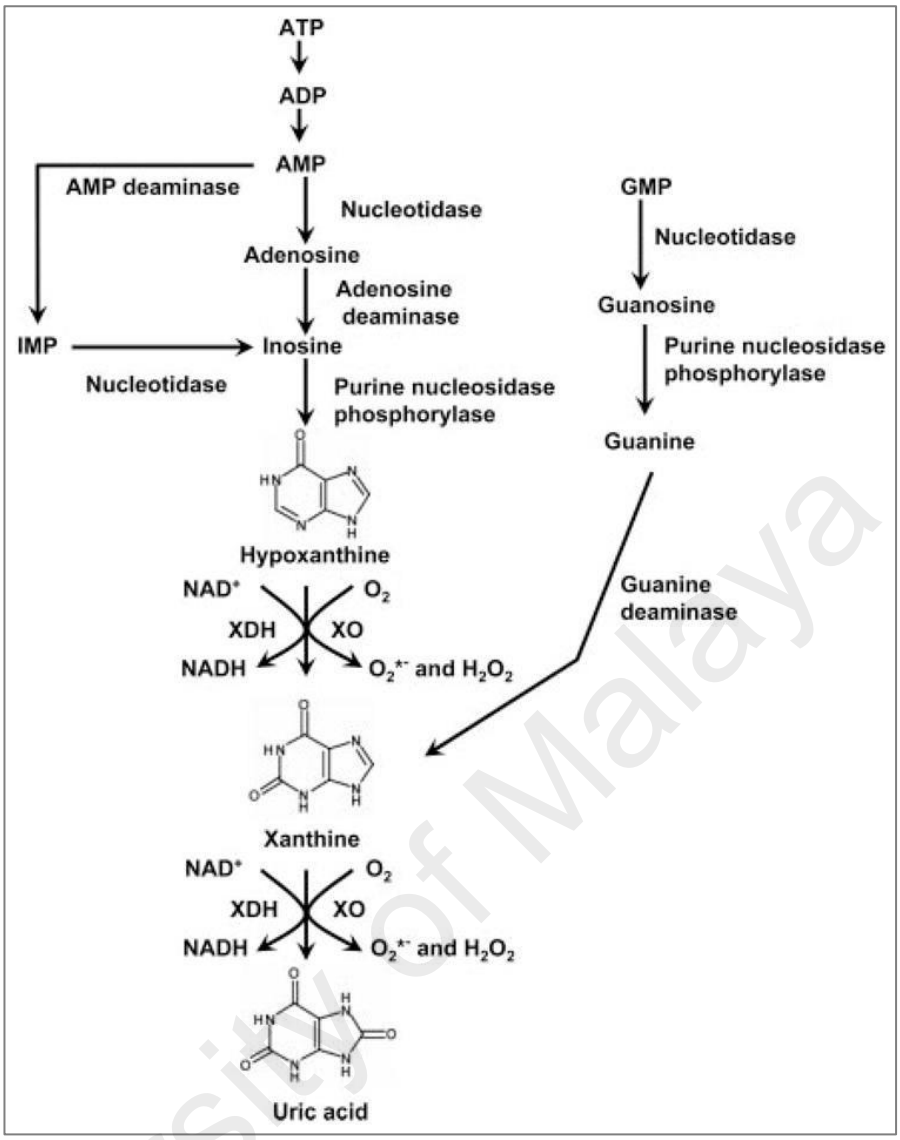

Figure 2.2: Schematic diagram of the purine degradation pathway (Pacher *et al.*, 2006)

Because molecules that contain purines are crucial for survival, vertebrates, including humans, have established a powerful system to generate enough purine nucleosides to be utilized in metabolism using materials such as glucose, glycine, and glutamine that are easily available in the body. Apart from that, purine nucleosides can also be recovered by obtaining it from the diet or from the body itself. Mammals get rid of the excess purine nucleosides by breaking it down in the liver and then expelled by the kidneys (Ishikawa *et al.*, 2013).

2.1.1 Xanthine oxidase

Xanthine oxidase exists as an active form homodimer having a molecular mass of approximately 290 kDa, where each of the identical monomer subunit (approximately 145 kDa) performs in independent manner during catalytic reaction (Lin *et al.,* 2002). Every subunit is made up of an N-terminal 20-kDa domain that contains two iron-sulfur clusters, a central 40-kDa flavin adenine dinucleotide (FAD)-binding domain and a C-terminal 85-kDa molybdopterin-binding domain with the four redox centers positioned in practically linear manner. It is an important enzyme that performs catalytic reaction in terminal two steps of purine catabolism in humans (Borges *et al.*, 2002).

Xanthine oxidase appears as molybdopterin-containing flavoproteins that carry out the catalytic hydroxylation of hypoxanthine to xanthine, and subsequently xanthine to uric acid (Pacher *et al.,* 2006). Uric acid is the final compound in the pathway (Doehner *et al.,* 2016). Xanthine oxidase can be found in wide range of organs such as kidney, liver, lung, gut, brain, heart, and also plasma (Pacher *et al.,* 2006). Three main organs that play crucial role in metabolism and excretion are kidney, liver, and lung (Borges *et al.*, 2002).

Aside from being a housekeeping enzyme, xanthine oxidase acts as rate-limiting enzymes in purine nucleotide degradation. It is represented by two structures which are xanthine oxidase (XO) and xanthine dehydrogenase (XDH) (Cantu-Medellin & Kelley, 2013). Both xanthine oxidase and xanthine dehydrogenase presents as interconvertible forms of the same enzyme (Pacher *et al.,* 2006). Xanthine oxidase favors oxygen (O_2) whereas xanthine dehydrogenase favors NAD^+ as electron acceptors. As a terminal electron acceptor, oxygen is bonded to electrons in unstable manner generating hydrogen peroxide (H_2O_2) and superoxide anion (O^-) spontaneously. Xanthine dehydrogenase undergoes oxidation to form uric acid, while the electrons move through

Fe/S transporters to $FADH_2$. In the process, NAD^+ is reduced to NADH. When xanthine oxidase is oxidized to uric acid, the electrons are transported to FAD and O_2 is transformed into H_2O_2 and O^- by reduction reaction (Cantu-Medellin & Kelley, 2013). Xanthine oxidase that comes from mammals, including humans, is produced in the form of dehydrogenase. However, they are easily transformed into oxidase form via oxidation of sulfhydryl residues or proteolysis (Pacher *et al.,* 2006). Figure 2.3 shows the conversion of xanthine dehydrogenase and xanthine oxidase to uric acid.

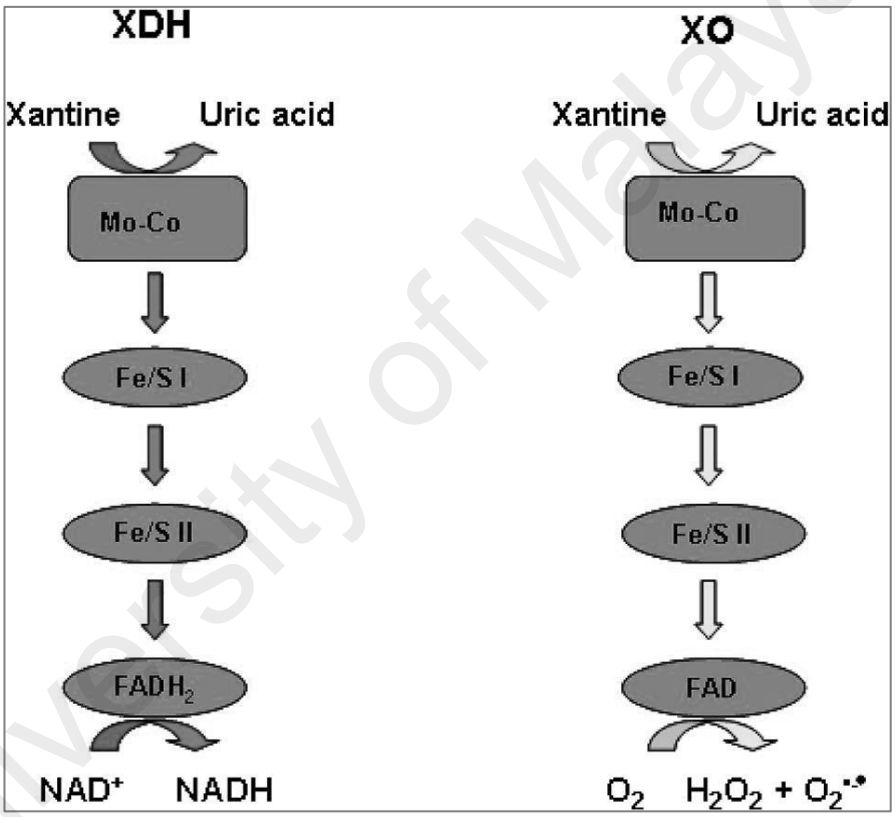

Figure 2.3: Oxidation of XDH (left) and XO (right) (Maiuolo *et al.,* 2016)

Xanthine oxidase is regarded as a key donor of free radicals that lead to in many pathological conditions (Mamat *et al.,* 2014) especially during exercise (Spanou *et al.,* 2012). This is because the catalytic oxidation of xanthine oxidase generates superoxide radicals during the enzymatic process (Chu *et al.,* 2014). Excessive reaction of xanthine

oxidase causes increased amount of uric acid which consequently increase the accumulation of uric acid in receptive tissues, and hence prompts the inflammatory pathways along with the release of reactive oxygen species (Pacher *et al.,* 2006). It can causes oxidative damage to existing tissues that are essential in numerous pathological activities like cancer, aging, inflammation and atherosclerosis (Sweeney *et al.,* 2001).

Remarkably, the first biological system to be recognized in generating reactive oxygen species (ROS) is xanthine oxidase (McCord, 1968). Xanthine oxidase also contributes as the role of major ROS producing source in human body where ROS are manufactured stoichiometrically by the enzymatic reaction of xanthine oxidase (Doehner *et al.,* 2016). As stated by Scott *et al.* (2013) the elevated level of ROS in human body system may exhibit broad variety of damaging effects over long-term periods. However, in the circumstances of the inflammatory response, xanthine oxidase is believed to fight the attacks of harmful pathogens by producing ROS and can act as the medium of innate immunity (Martin *et al.,* 2004).

2.1.2 Uric acid

Production of uric acid is occurs predominantly in the liver and it is discarded into the urine by kidneys (Hayashi *et al.,* 2000). Represented by molecular formula of $C_5H_4N_4O_3$ uric acid (7,9-dihydro-1H-purine-2,6,8(3H)-trione) is a heterocyclic organic compound having a molecular weight of 168 Da. Uric acid is a weak acid physiologically representing a pKa of 5.8 where it mainly appears as the salt of uric acid, urate. When the concentration of urate escalates, the production of uric acid crystal escalates. Normal uric acid indicator in human blood is 2.5 to 7.0 mg/dL for male and 1.5 to 6.0 mg/dL for female. Uric acid possesses low solubility in water, where its average concentration in human blood is near to solubility limit (6.8 mg/dL). As the uric

acid quantity increases more than 6.8 mg/dL, uric acid crystals become monosodium urate (MSU) (Jin *et al.,* 2012). The concentration of solubility of monosodium urate is dependent on diet, body weight, age, pH, and temperature (Campion *et al.,* 1987). In the majority of mammals, the conversion of purine into intermediate uric acid is assisted by enzyme uricase in which the intermediate uric acid is then converted into a compound called allantoin. Allantoin is a compound that has a very high solubility. It can move through the bloodstream and discarded out of the body after being filtered by the kidneys (Ishikawa *et al.,* 2013). On the contrary, because humans are lacking of enzyme uricase, we are unable to oxidize uric acid into allantoin and therefore, uric acid is largely discarded by kidneys everyday (Jin *et al.,* 2012).

Uric acid levels in blood are influenced by two aspects. First is the proportion of uric acid synthesis in the liver and second is the rate of uric acid discharge from the kidneys (Ishikawa *et al.,* 2013). The making of uric acid and its degradation process are complex because many factors that control hepatic production as well as uric acid excretion of renal and gut are involved. The exogenous pool of purines and endogenous purine metabolism contribute to the production of uric acid as an end product. The exogenous pool differs outstandingly according to the amounts of purines obtained from the diet as well as amounts of purines synthesized from the proteins of animal. On the other hand, the sources of endogenous uric acid formation are from liver, intestines, and tissues like vascular endothelium, kidneys, and muscles (Chaudhary *et al.,* 2013). Excretion of uric acid from kidneys possesses an outstanding impact on the level of uric acid in blood (Ishikawa *et al.,* 2013). The rise of serum uric acid level give rise to the occurrence of a few disorder related to oxidative stress (Haidari *et al.,* 2011).

2.2 XO-related diseases

The capability of xanthine oxidase in generating ROS be the cause of increasing interest in the enzyme as being the starter of tissue injury in many pathological conditions (Bonomini *et al.,* 2008). Constant exposure to contaminants and chemicals results elevated level of free radicals in the body, which are considered as the principal reason behind the emerge of different diseases and disorders (Cheng *et al.,* 2003).

Xanthine oxidase causes diverse ischemia-reperfusion injury in tissues, vascular injuries, and inflammation. Apart from that, its action gives an exceptional impact on viral infection, oxidative stress, respiratory syndrome, thermal stress, brain edema, and hemorrhagic shock *in vivo* (Naoghare e*t al.,* 2010).

According to Boban *et al.* (2014) xanthine oxidase is also implicated in cellular aging, atherosclerosis, hypertension, and metabolic disorders. Borges *et al.* (2002) added that xanthine oxidase activity has distinctly leaded to myocardial infarction, diabetes, and cancer. Pacher *et al.* (2006) stated that mutagenesis and hepatitis are the diseases linked by elevated xanthine oxidase serum level. It is found that level of serum uric acid level is usually rose in heart failure with strong relation to heart failure severity according to NYHA (New York Heart Association) or the capacity of spiroergometry exercise (Doehner *et al.,* 2001). Increased level of uric acid is proven in few studies to be a powerful and autonomous predictor of death in heart failure patients (Doehner *et al.,* 2016). Song *et al.,* (2015) stated that chronic kidney disease and cardiovascular disease are the outcome from xanthine oxidase activities. Nevertheless, above all, excessive uric acid level in the blood contributes to the development of hyperuricemia, a clinical complication that causes gout disease (Lü *et al.,* 2013).

2.2.1 Hyperuricemia and gout

According to Gliozzi *et al.* (2016) hyperuricemia and gout are pathological disorder distinguished by excessive making and insufficient excretion of uric acid, the terminal output of purine metabolism pathway discharged through the urine. It is primarily due to metabolic disorder on uric acid production or excretion in the body (Haidari *et al.,* 2008). Apart from gout, recent research showed that asymptomatic hyperuricemia is connected to cardiovascular diseases (Agabiti-Rosei & Grassi, 2013) as well as tumor lysis syndrome (TLS) and chronic kidney disease (CKD) (Edwards, 2008). Roughly 90% of hyperuricemia occurrence is associated with damaged renal excretion (Choi & Ford, 2007). Edwards (2008) stated that apart from gouty arthritis, hyperuricemia is an exceptionally well-verified reason for kidney stones development in uric acid and acute kidney failure. Factors that play part in triggering hyperuricemia are insulin opposition, obesity, consumption of alcohol, use of diuretic, genetics, hypertension, renal insufficiency, and others (Yamamoto *et al.,* 2005).

Gout or gouty arthritis is a metabolic disorder that occurs when elevated concentration of uric acid present in the blood develop microscopic crystals in the joint after a period of time. This as a consequence, results in acute inflammatory arthritis (Janssens *et al.,* 2006) generally due to xanthine oxidase over-activity in purine degradation (Burke *et al.,* 2006). The accumulation of the monosodium urate crystals (needle shaped) in the synovial fluid of the major joints causes an utmost painful critical arthritis accompanied with periodic gout attacks (Rang *et al.,* 2001) such as painful inflammation, swelling and erythema (Irondi *et al.,* 2016). Two types of gout are acute gout and chronic gout, where chronic gout is always connected by hyperuricemia (Ling & Bochu, 2014). The involvement of the sudden attacks in gout frequently occurs in the joint at the bottom of the big toe and often happens when the body temperature is at its lowest degree during early hours of a day. Despite the fact that the severe strike of gout

is short-lived and diminish spontaneously, the effect can momentarily weaken that particular person and increase susceptibility of the next attacks (Becker *et al.,* 2005). Lin *et al.* (2013) revealed that gout may lead to deformities on ocular surface for example vascular changes, tophi deposition, and subconjunctival transparent vesicles.

Gout is one of the most prevalent diseases that give impact on human and its incident keep on growing compared to the last few decades (Sowndhararajan *et al.,* 2012). According to Richette & Bardin (2010) not more than 2% of adults in industrialized nations are affected by gout and this ailment denoted as the most recurrent case of inflammatory arthritis in men. The burden of gout is increasing globally, with the prevalence recorded at 0.08% (Smith *et al.,* 2014). The prevalence of gout rises with age in all countries where the data reaches a plateau for individuals older than 70 years old, meanwhile in terms of sex, men exhibited higher gout prevalence than women (Kuo *et al.,* 2015). The manifestation of gout disease is due to a person's lifestyle and diet. Heavy alcohol drinks such as beer and liquor, and foods rich in nucleic acids such as meats and seafood impart a great influence on development of gout (Saag & Mikuls, 2005). High consumption of fructose also reported to be one of the risk factors of gout (Dubchak & Falasca, 2010). Three phases for gout management are: (i) healing the acute attack; (ii) reducing the uric acid level to obstruct eruption of gouty arthritis and deposition of urate crystals in tissues; and (iii) preventive treatment to avert acute flares (Schlesinger, 2004). Therefore, one way to cure gout is by utilizing a compound that has the capability to enhance the excretion of uric acid or suppress the biosynthesis of uric acid (Umamaheswari *et al.,* 2009).

2.3 Xanthine oxidase inhibitors

Since xanthine oxidase is the key enzyme that fundamentally generates uric acid in purine catabolism, it is regarded as the most assuring goal in the therapy of hyperuricemia and gout (Lü *et al.,* 2013) by acting like a mediator that can effectively decrease the activity of xanthine oxidase (Becker *et al.,* 2005) and suppress uric acid generation (Borges *et al.,* 2002). Allopurinol and febuxostat are the drugs that possess antihyperuricemia effect and able to competitively retard the activity of xanthine oxidase (Kumar & Azmi, 2014). Another new drug for hyperuricemia therapy is topiroxostat (Ohya & Shigematsu, 2014) and pegloticase (Burns & Wortmann, 2012). The investigation of possible inhibitors of enzyme in natural or artificial sources is a crucial stage to uncover potential drugs and its process of development. Apart from its inhibitory capability, the compounds should manifest a strong selectivity towards targeted enzyme so that the desired outcome can be achieved even with low dosage (Rodrigues *et al.,* 2016). Figure 2.4 shows the chemical structures of allopurinol and febuxostat as xanthine oxidase inhibitors.

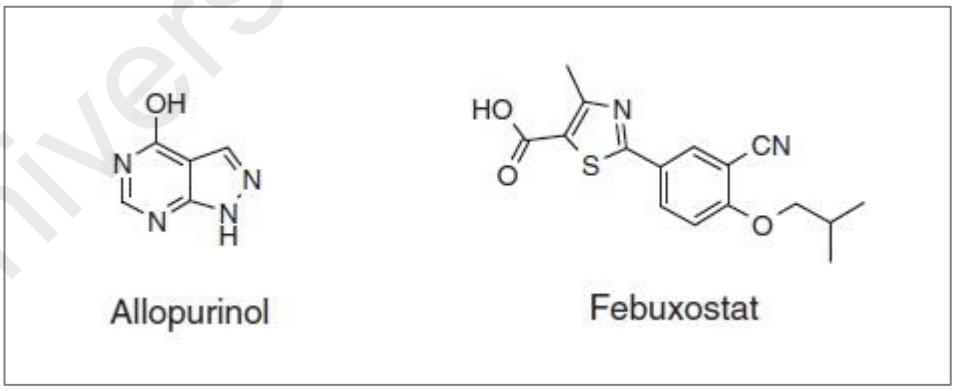

Figure 2.4: Chemical structures of allopurinol and febuxostat (Song *et al.,* 2016)

2.3.1 Allopurinol

Allopurinol (1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one) is a xanthine oxidase inhibitor that owns structure similar to purine (Wortmann, 2005). It is the first-line drug for treating hyperuricemia and its complications (Wu *et al.,* 2015). Allopurinol acts by blocking xanthine oxidase activity in the final step of uric acid biosynthesis, thus reducing the serum urate level. Clinically, the use of allopurinol has been employed for more than four decades (Lü *et al.,* 2013) and first approved by Food and Drug Administration (FDA) to decrease serum urate level (Emmerson, 1996). The efficacy of allopurinol is dose-linked and to achieve the target urate level, doses higher than the often used prescription (300 mg/dL) is required (Reinders *et al.,* 2009). Allopurinol is the most common medication used in gout and tumor lysis syndrome (TLS) treatment. However due to allopurinol hypersensitivity and zero effect, zero endurance to the toxic effect of allopurinol, and drug-drug communication with allopurinol, refractory gout is initiated in patients (Mughal *et al.,* 2010). That being the case, combining a compound with allopurinol, and lessens the required high dose of allopurinol for hyperuricemia or gout therapy conveys a desirable potential in clinical field (An *et al.,* 2010).

Allopurinol is notably known to possess significant undesirable effects like renal damage and skin reactions due to its administration (Doehner *et al.,* 2016). Superoxide radicals are produced from the reaction involving allopurinol (Berry & Hare, 2004). The most common impacts of allopurinol are hypersensitivity response, gastrointestinal disturbance, and skin rash, where the reaction of hypersensitivity may reoccurring within months and years of taking the drug. Patients that suffer reduced renal functions, with constant allopurinol dosage are generally susceptible to these adverse effects (Pacher *et al.,* 2006). According to Tsai & Yeh (2010) the other life-threatening effects of allopurinol hypersensitivity syndrome are fever, hepatitis, and eosinophilia. Other adverse effects of allopurinol are Toxic Epidermal Necrolysis syndrome (TENS) and

Stevens-Johnson syndrome (SJS) (Halevy *et al.,* 2008). Approximately 44 cases of Stevens-Johnson syndrome emerged in every million new patients treated with allopurinol (Kim *et al.,* 2013). These serious cutaneous adverse reactions (SCARs) of allopurinol usually appear in the range of three months after the treatment or increased dose initiation (Reinders *et al.,* 2009). The adverse effects of allopurinol result in the termination of the therapy in not more that 5% patients, and analysis made by several studies proposed that the level of cooperation of the drug therapy with patients is only about half of the periods of treatment (Riedel *et al.,* 2004).

2.3.2 Febuxostat

Febuxostat is a novel nonpurine specific inhibitor of xanthine oxidase (both oxidized and reduced form) which is approved by US Food and Drug Administration (FDA) in February 2009 to manage hyperuricemia in individuals that suffer from gout disease (Ernst & Fravel, 2009). Febuxostat is believed to act as a better potential alternative to allopurinol due to the fact that it possess the least possible effects on enzymes encompassed in purine metabolism pathway and it is metabolized by the oxidation reaction in the liver and formation of glucuronide (Becker *et al.,* 2005). For a patient with hyperuricemia, febuxostat is a potentially better alternative than allopurinol (Tojimbara *et al.,* 2014) and according to Ernst & Fravel (2009) febuxostat has been successfully used in individuals that experience intolerance towards allopurinol. Report findings showed that most patients have high tolerance to febuxostat (Terkeltaub, 2010). Ye *et al.* (2013) stated that febuxostat is safe to be used in patients that have mild to moderate renal injury.

For the treatment of individuals with asymptomatic hyperuricemia, the use allopurinol and febuxostat are not advised due to the fact that they exhibit possible side

effects (Khanna *et al.,* 2012). The adverse events that could occur from the administration of febuxostat are skin rash, nausea, arthralgias, and escalated liver enzyme. In addition, the administration of febuxostat should be partly covered by colchicines or NSAID (Nonsteroidal Anti-inflammatory drugs) initially because acute flares may arise during the beginning of the treatment (Ernst & Fravel, 2009). In patients with CKD (Chronic Kidney Disease) the side effect of neutropenia was reported due to the use of febuxostat (Kobayashi *et al.,* 2013). Febuxostat showed moderate increasing risk of skin reactions in individuals with an experience of cutaneous adverse events with allopurinol (Bardin *et al.,* 2016). Study by Faruque *et al.* (2013) revealed that even though febuxostat is connected to display high probability of getting ideal serum urate level of <6.0 mg/dL, the pooled results showed significant heterogeneity. Clinically, there is no verification that febuxostat is better than allopurinol and since febuxostat is higher in cost, they suggested that it should not be regularly used for individuals with chronic gout.

2.3.3 Natural alternatives

Since ancient times, plants have been extensively utilized in almost all medicinal therapy until the advance of synthetic drugs begins in 19th century (Zheng & Wang, 2001). A number of natural products show potent effects in reducing uric acid level, thereby recover hyperuricemia with hepatorenal dual regulation (Hao *et al.,* 2016). Regardless of the present of drugs like allopurinol and febuxostat as xanthine oxidase inhibitors, allergic responses are triggered on certain patients because of these medications. Thus, researchers are focusing extensively on phytomedicines as xanthine oxidase inhibitors. For that reason, the utilization of medicinal plants becomes significant in the therapeutic cure of gout and other chronic diseases (Kumar & Azmi,

2014). These medicinal plants can undertake the role of natural gout medication, replacing other synthetic drugs (Azmi *et al.,* 2012). The advantages of using herbal medicines in curing diseases are less side effects, notable efficiency, comparative safety, and less expensive to those who cannot afford to have expensive medication (Sunmonu & Afolayan, 2013). However, the use of medicinal plants in modern treatment is afflicted by their insufficiency in scientific evidence (Zhu *et al.,* 2004). This is because medicinal plants were used based on the experience of old medicine system in curing and preventing hyperuricemia and gout (Kong *et al.,* 2000). Zhu *et al.* (2004) claimed that natural polyphenolic compounds like flavonoids exhibited the activity of xanthine oxidase inhibition in the form of purified extracts. Umamaheswari *et al.* (2009) stated that the presence of phenolic and flavonoid compounds in an extract may contribute to xanthine oxidase inhibitory activity. Phenolic compounds are secondary metabolites that are usually present in plants in which they are involved in antioxidant activity and beneficial to defend plants from the attack of pathogens and radiation (Tiveron *et al.,* 2012). Countries like India, Vietnam, Philippine and north east America own few medicinal plants that possess xanthine oxidase inhibitory effect (Wong *et al.,* 2014). It has been discovered that antioxidants obtained from plants exhibit antigout capacity. This is because from several *in vitro* studies that have been conducted, compounds like alkaloids (eg: piperine), phenolics (eg: caffeic acid), essential oils (eg: cinnamaldehyde), tannins (eg: gallic acid), flavonoids (eg: quercetin), coumarins (eg: fraxetin), and iridoid glucosides (eg: specioside) are likely to possess antigout potential by inhibiting xanthine oxidase activity (Ling & Bochu, 2014). Latest evidence showed that polyphenols exhibit dual effects which are inhibiting xanthine oxidase and scavenging free radicals. This proposes new discovery in therapeutic approach to diseases like hyperuricemia, oxidative stress triggered from uric acid, inflammation, and tissue damage (Gliozzi *et al.,* 2014).

2.4 Studied plant - *Pandanus amaryllifolius* Roxb.

P. amaryllifolius which is also known as 'daun pandan' or 'pandan wangi' belongs to the member of screw pine family Pandanaceae. It is a typical culinary plant distributed in South East Asia tropical region (Ooi *et al.,* 2004). In countries like Malaysia, Thailand, Indonesia, and India, this fragrant screw pine is cultivated extensively (Rayaguru & Routray, 2010). According to Callmander *et al.,* (2012) the family Pandanaceae world distribution includes Malaya, Borneo, Thailand, Philippines, India, Australia, Africa, New Guinea, Madagascar, New Caledonia, Solomon Islands, Sao Tomé Island (Figure 2.5). The taxonomy of *P. amaryllifolius* is as follow (APG III system 2009):

Table 2.1: Taxonomic position of *Pandanus amaryllifolius* Roxb.

Kingdom: Plantae
Angiospearmae
Monocots
Order: Pandanales
Family: Pandanaceae
Genus: *Pandanus*
Species: *Pandanus amaryllifolius* Roxb.

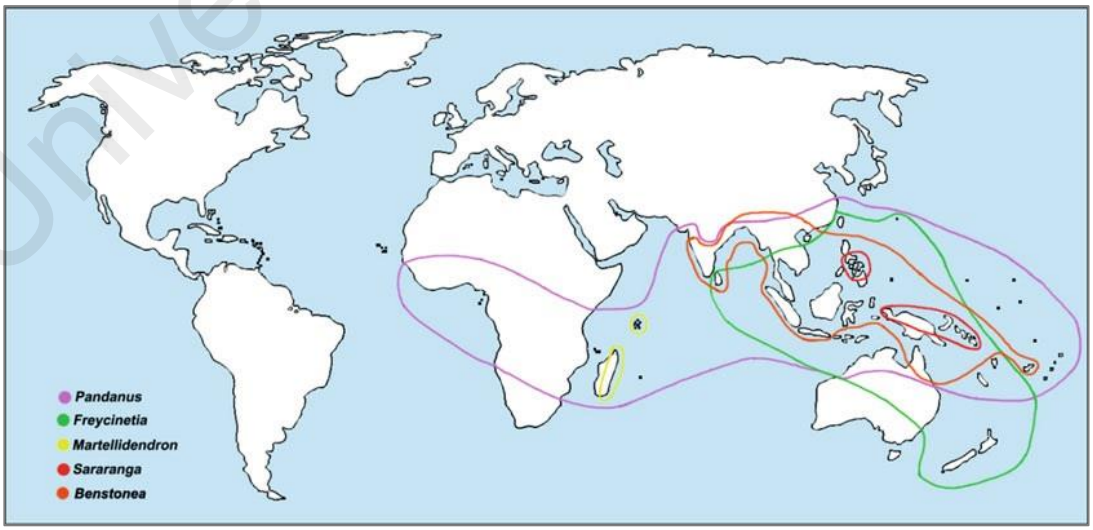

Figure 2.5: World distribution of family Pandanaceae (Nadaf & Zanan, 2012)

William Roxburgh is a botanist and Scottish surgeon that described *P. amaryllifolius* (Nadaf & Zanan, 2012). Genus *Pandanus* consists of roughly 700 species widespread in both tropical and sub-tropical areas (Tan *et al.*, 2012). Plants from genus *Pandanus* appear as evergreen trees or shrubs that are palm-like (Bhattacharjee *et al.*, 2005). The leaves of pandan are green in colour, has narrow and strap shape appearance and spirally arranged at apex of stems (Figure 2.6) (Peter, 2012). The blades of *P. amaryllifolius* leaves usually 25–75 cm in length, 2–5 cm in width (small form) and 150–220 cm in length, 7–9 cm in width (large form), seldom with 1–3 little stout prickles on midrib adjacent to the base (Wakte *et al.*, 2009). The stem of *P. amaryllifolius* is slender roughly 2–5 cm in thickness. Its height can extend up to 4.5 m, if appropriate support is given and factors that lead to prostration is absent. Two categories of height are up to 1.6 m (small form) and up to 4.5 m (large form) (Wakte *et al.*, 2007). The growth forms of *P. amaryllifolius* are depicted in Figure 2.7.

Figure 2.6: *Pandanus amaryllifolius* Roxb. (Zanan *et al.*, 2016)

Figure 2.7: Distinct small and large forms of *P. amaryllifolius* (Wakte *et al.*, 2009)

P. amaryllifolius is the sole species of family Pandanaceae that owns fragrant leaves. The main compound responsible in representing aromatic feature in *P. amaryllifolius* is 2-acetyl-1-pyrroline (2AP) (Yahya *et al.*, 2010). Quantitatively, 2AP present in this plant possesses the highest amount among the plant kingdom. This compound is the

similar to the ones present in aromatic rice like Jasmine and Basmati rice. For that reason, pandan leaves are used in cooking plain unscented rice to mimic aromatic rice varieties which are a lot pricey, as a substitute (Ravindran & Balachandran, 2005). Essential oils present in pandan leaves convey tenfold more flavor than aromatic rice (Rayaguru & Routray, 2010). Pandan leaves are used in large-scale in cooking because of its natural food dye and its scent (Han *et al.,* 2014). Abundant chlorophyll present in the leaves provides green colour to foods and drinks (Zanan *et al.,* 2016). The extracts of pandan leaves are used as an essence in bakery (Bhattacharjee *et al.,* 2005) as a substitute to vanilla essence (Wyk, 2005). The powdered form of green pandan leaves is safe to use as food additive (Porrarud & Pranee, 2010). Occasionally, the leaves are put into frying oils in order to transmit its flavor to the fried food (Nor *et al.,* 2008). At times, pandan leaves are added in cold drinks made from unripe coconut water as well as to custards and sweetened puddings made from sticky glutinous rice (Bhattacharjee *et al.,* 2005). In Malaysia, pandan leaves are used to make coconut jam, candies, ice cream, and desserts (Ghasemzadeh & Jaafar, 2014). It can also be used in flavouring meat and vegetables or blend with sauces to enhance the taste (Rayaguru & Routray, 2010). According to Ravindran & Balachandran (2005) the famous breakfast menu in Malaysia, 'Nasi Lemak' is a traditional dish made by mixing the rice with 'santan' (coconut milk) and pandan leaves that are soaked during the cooking process.

Apart from its culinary importance, pandan is utilized as ornamental plants as well as grown in kitchen garden due to its ability to live in wild condition (Rayaguru & Routray, 2010). Pandan leaves can also be implemented as a pest management agent because it is natural and environmentally friendly, for instance, taxi drivers in Malaysia and Singapore are said to put *P. amaryllifolius* leaves in their taxi to fend off cockroaches. *P. amaryllifolius* possess secondary value in intensifying visual and olfactory pleasure to humans. In Malaysia, pandan leaves is used traditionally as

medicinal bath and hair wash to mothers after giving birth. 'Potpourri' which is a mix of chopped pandan leaves with the petals of several sweet-smelling flowers is used in traditional celebrations in Malaysia. In Philippines, pandan leaves are used to prepare a lotion along with vinegar and ash in healing measles, leprosy, sore throat, and as laxatives (Samy *et al.,* 2005).

Apart from being utilized in perfume-making industry, *P. amaryllifolius* possesses antidiabetic, cardio-tonic, and diuretic (Wakte *et al.,* 2010) as well as antispasmodic, and stimulant properties (Quisumbing, 1978) in medical field. *P. amaryllifolius* leaves are used in refreshing the body, curing fever, and healing indigestion (Cheeptham & Towers, 2002). It is also used to heal problems related to thyroid (Jong & Chau, 1998). Pandan leaves are immersed in coconut oil for a few days and later the oil is used to heal rheumatism. Leaves infusion is obtained internally to relieve restlessness. In Thailand, *P. amaryllifolius* is used traditionally to combat diabetes (Ravindran & Balachandran, 2005). According to Ooi *et al.* (2004) unglycosylated pandamin protein is present in the pandan leaves which express antiviral mechanism against influenza virus and human virus, herpes simplex virus type-1. Ghasemzadeh & Jaafar (2014) stated that *P. amaryllifolius* shows antioxidant bioactivities. Study made by Nor *et al.* (2008) reported that ethanol extract of *P. amaryllifolius* grown in Malaysia displayed an exceptional heat-stable antioxidant feature. The content of *P. amaryllifolius* includes quercetin (Miean & Mohamed, 2001), alkaloids such as Pandanusine A (Cheng *et al.,* 2017), fatty acids and esters (Zainuddin, 2004), tocopherols, tocotrienols, and carotenoids (Lee *et al.,* 2004) and non-specific lipid transfer proteins (Ooi *et al.,* 2006).

2.4.1 Phytochemical study on *P. amaryllifolius*

Medicinal plants possess phytochemical compounds which are crucial in exerting health goodness or improving state of ailment. However, numerous phytochemicals are still unidentified (Boyer & Liu, 2004). Study by Ghasemzadeh & Jaafar (2013) showed that *P. amaryllifolius* leaves have good bioactive compounds such as phenolic acids (gallic acid) and flavonoids (catechin, kaempferol and naringin). These bioactive compounds showed synergistic ability in obstructing breast cancer cell proliferation and could be the prospective natural food additive. Research by Wakte *et al.* (2012) showed that out of 31 volatiles, five major volatile compounds detected in *P. amaryllifolius* are 2AP, nonanal, phytol, 2-hexenal, 2,6-nonadienal and hexanal. Dominant components of essential oils obtained from the leaves are phytol, squalene, pentadecanal, pentadecanoic acid, 3,7,11,15-tetramethyl-2-hexadecen-1-ol, and phytone (Chen & Ge, 2014). Main carotenoids detected in *P. amaryllifolius* leaves are β-carotene and lutein. Together with carotenoids precursors which are α-carotene and β-carotene, three norisoprenoids discovered are α-ionone, β-ionone, and β-cyclocitral (Ningrum *et al.*, 2015). Study by Cheng *et al.* (2017) revealed seven latest alkaloids from *P. amaryllifolius* ethanolic extract which are pandanusine A (1), pandanusine B (2), pandalizine C (3), pandalizine D (4), pandalizine E (5), norpandamarilactonine C (6), and norpandamarilactonine D (7) (Figure 2.8).

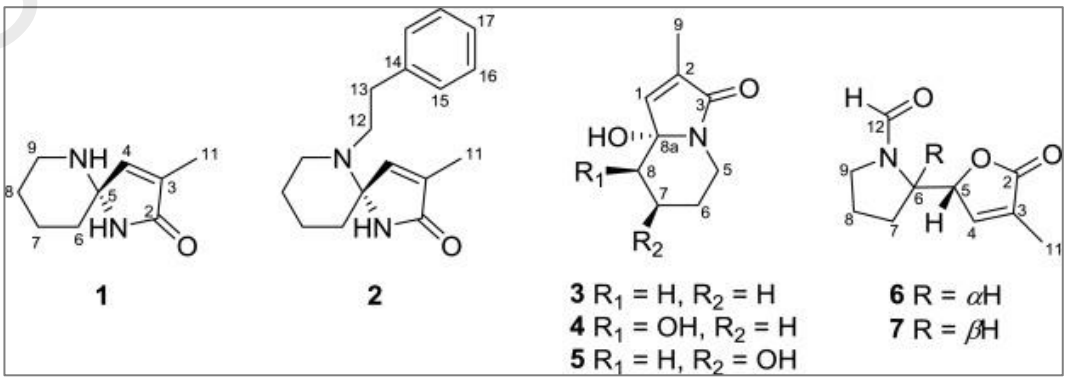

Figure 2.8: Structures of new alkaloids (Cheng *et al.*, 2017)

2.5 Antioxidant

Antioxidant is a substrate that prevents or delays the oxidation of another substrate. It often exists in a very low concentration (Halliwell & Gutteridge, 1989). Being the fundamental role in keeping the body healthy, the main sources of naturally existing antioxidants are commonly derived from vegetables, fruits, and whole grains. Vitamin C, Vitamin E, phenolic acids, carotenes, phytate and phytoestrogens from plants have a potential to decrease risk of diseases. In normal diet, almost all antioxidants are obtained from plants where the antioxidant compounds are categorized into assorted classes representing an assortment of physical and chemical properties (Prakash, 2001). Consumption of vegetables and fruits is linked to low chance of cancer and cardiovascular diseases (Cazzi *et al.*, 1997).

The amount of phenolic compounds in plants is ubiquitous. They appear as a crucial contributor of colour and flavour in numerous fruits and vegetables. They exist as C15 compounds with two phenolic rings linked by a 3-carbon unit. They are grouped based on the different substituent present on the rings and the level of ring saturation. Often, to raise their solubility in water, they are chained to sugar moiety (Lin *et al.*, 2001). Compounds like gallates have strong antioxidant capability whereas compounds like mono-phenols are weak antioxidants (Prakash, 2001). Phenolic compounds possess antimicrobial, anti-inflammatory, antithrombotic, antiallergenic, antiartherogenic, vasodilatory and cardioprotective effects (Alpinar *et al.*, 2009). Because of its conjugated rings and hydroxyl groups, plenty of phenolic compounds are able to potentially function as antioxidants in oxidative reaction by stabilizing or scavenging free radicals better than vitamin C and vitamin E (Seyoum *et al.*, 2003). According to Nijveldt *et al.* (2001) polyphenols play essential part in redox reaction that result in free radical neutralization, oxygen relieve, and peroxides decomposition.

Free radicals are continuously generated because the body response towards internal and external impulse (Matés *et al.*, 1999). These radicals, in low quantity, play crucial role in controlling growth, signaling transducers, and being the members of immune system (Atmani *et al.*, 2009). In spite of that, elevated free radicals level results in oxidative stress which as a consequence, gives rise to the emerge of diseases like ageing, cancer, arteriosclerosis, and neurodegenerative diseases (de Oliveira *et al.*, 2009). Even with the available of synthetic drugs which are much stable and potent, there is limitation for the usage of the drugs because of potential side effects that could occur from its activity (Pokorný, 2007). Antioxidants possess high effectiveness in scavenging free radicals and able to extinguish reactive oxygen species by the means of scavenging radicals that are about to appear, interfering chain reactions, and attachment of metal ions (Shah *et al.*, 2014). The synergistic interaction of antioxidants shows moderate effects in preventing the oxidative damage of body cells (Karou *et al.*, 2005).

CHAPTER 3: MATERIALS AND METHODS

3.1 Plant sample

1.2 kg of fresh *Pandanus amaryllifolius* Roxb. plants were collected from Institute of Biological Sciences garden, University of Malaya, Kuala Lumpur in March 2015 and authenticated by plant taxonomist Professor Dr. Ong Hean Chooi from Institute of Biological Sciences, University of Malaya. A voucher specimen of *P. amaryllifolius* with voucher no.: KLU 49087 was deposited in the Herbarium of University of Malaya, Kuala Lumpur. All parts of the plants including the roots were rinsed and air-dried at room temperature for approximately three weeks until completely dried. The 417 g of dried samples were then ground into fine powder using a blender.

3.2 Chemicals and reagents

Bismuth nitrate, potassium iodide, vanillin, sodium carbonate, sodium nitrite, sodium acetate, sodium hydroxide, sodium chloride, aluminum chloride, ferrous sulfate, ferric chloride, ferrous chloride, monopotassium phosphate, dipotassium phosphate, gallic acid, quercetin, ascorbic acid, hexane, petroleum ether, chloroform, methanol, ethanol, acetone, and glacial acetic acid, were purchased from Chemolab Supplier (Malaysia). Ethylenediaminetetraacetic acid disodium salt dehydrate, DMSO, hydrochloric acid, and sulphuric acid were purchased from Merck Chemical Co. (Malaysia). DPPH, TPTZ, ferrozine, xanthine, xanthine oxidase (source: bovine milk), allopurinol, oxonic acid potassium salt, ρ-anisaldehyde, Folin-Ciocalteu reagent, and hydrogen peroxide solution were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

3.3 Instruments

Perkin Elmer Clarus 600 Turbo Mass GC-MS (Perkin Elmer, USA), Agilent 6550 iFunnel Q-TOF LC/MS (Agilent Technologies, USA) with C18 column (2.1 mm × 100 mm, 1.8 μm), UV-vis spectrophotometer-1700 (Shimadzu, Japan), and microplate reader (Tecan Sunrise, Austria).

3.4 Preparation of plant extracts

The extraction of phytochemical compounds in *P. amaryllifolius* was carried out by soaking 25 g dried powder in hexane, petroleum ether, chloroform, methanol, and distilled water respectively with the ratio of 1:10 (1 g of powder per 10 mL of solvent). The mixture was kept in water bath at 40°C for 8 hours in a closed system sealed with parafilm, occasionally shaken. The mixture was then filtered with filter paper. The filtered mixture was concentrated by rotary evaporator under reduced pressure at 40°C to maintain phytochemical compounds from being destroyed. The crude water extract was obtained through freeze-drying method. The crude extracts; *P. amaryllifolius* hexane extract (PA-H), *P. amaryllifolius* petroleum ether extract (PA-PE), *P. amaryllifolius* chloroform extract (PA-C), *P. amaryllifolius* methanol extract (PA-M), and *P. amaryllifolius* water extract (PA-W) were kept in airtight container until further used.

3.5 Identification of the phytochemical compounds

3.5.1 Thin layer chromatography (TLC)

Thin layer chromatography was conducted to detect chemical compounds present in *P. amaryllifolius* extracts. Firstly, developing chamber was constructed by adding solvent system into the chamber where the depth should not exceed 0.5 cm. TLC was performed on silica gel, 60 F_{254} aluminium backed plates (size 8 cm × 2 cm) by placing the extract as a band on the plate using capillary tube. PA-H, PA-C, and PA-M were separated using solvent hexane: acetone (7:3), PA-PE was separated using solvent hexane: acetone (8:2), and PA-W was separated using chloroform: methanol (1:9). Plates with the extracts were placed inside the developing chamber and separation was developed. Then, the plates were dried and subjected to UV light at 254 nm and sprayed with Dragendroff's, vanillin-sulphuric acid and anisaldehyde-sulphuric acid reagents to detect the phytochemicals. Retention factor (R_f) value for each detected compound was calculated and recorded. R_f value is the ratio between the distance moved by the sample and the distance moved by the solvent. It is used to explain sample characteristics.

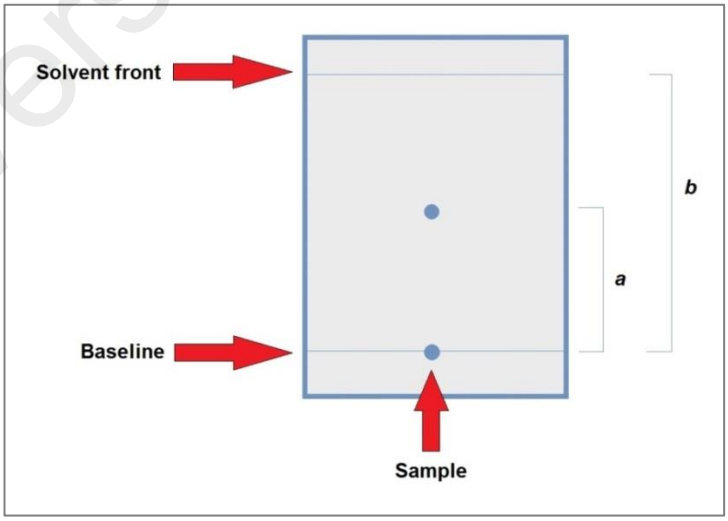

Figure 3.1: Determination of R_f value on TLC plate (Millar, 2012)

$$\text{Retention factor } (R_f) \text{ value} = \frac{\text{Distance travelled by sample (a) cm}}{\text{Distance travelled by solvent (b) cm}}$$

3.5.2 TLC spray reagent

(a) ***Dragendroff's reagent***

The application of Dragendroff's reagent on TLC chromatograms is to detect the presence of alkaloids in the sample. The spray reagent was prepared by preparing solution A (0.85 g of bismuth nitrate dissolved in 10 mL of glacial acetic acid and 40 mL of distilled water) and solution B (8 g of potassium iodide dissolved in 30 mL of distilled water). After that, 25 mL of solution A, 25 mL of solution B, 100 mL of glacial acetic acid, and 500 mL of distilled water were mixed to make the spray reagent. Dragendroff's reagent is stable and can be refrigerated for a few weeks. The presence of alkaloids was detected by observing orange spot on the TLC plates.

(b) ***Vanillin-sulphuric acid reagent***

Vanillin-sulphuric acid reagent was used to detect the presence of terpenoids and phenols in the sample. The spray reagent was made by adding 1 g of vanillin into 1 mL of concentrated sulphuric acid. The solution was then mixed with 100 mL of ethanol. The TLC plates were then sprayed with the vanillin-sulphuric acid reagent and heated on a hot plate for about 5–10 minutes at 100°C until the colour developed. The presence of terpenoids and phenols were detected by observing purple (terpenoids) and green (phenols) spot on the TLC plates.

(c) ***Anisaldehyde-sulphuric acid reagent***

Anisaldehyde-sulphuric acid reagent is able to detect the presence of phenolic compounds in the sample. It is prepared by mixing 0.5 mL of anisaldehyde, 10 mL of glacial acetic acid, 5 mL of concentrated sulphuric acid, and 85 mL of methanol together. The plates were then sprayed with the reagent and heated for 5–10 minutes on a hot plate at 100°C. The presence of phenols was detected by observing violet, blue, red, grey or green colour. Green colour represents flavonoids.

3.5.3 Gas chromatography mass spectrometry (GC-MS)

Crude PA-W and PA-M were subjected to GC-MS analyses. Out of five extracts available, only PA-W and PA-M were suitable to be used in specific column in GC-MS machine. The crude *P. amaryllifolius* extracts were sent to Institute of Systems Biology (INBIOSIS), Universiti Kebangsaan Malaysia (UKM), Selangor for analysis. Derivation of sample was conducted based on method by Azizan *et al.* (2012) using 40 µL of 20 mg/mL *O*-methoxyamine hydrochloride solution in anhydrous pyridine to the dried extracts. After the mixture was mixed vigorously, it underwent the incubation period of 30 minutes at 60°C in a heating block. Afterward, 40 µL of *N*-methyl-*N*-(trimethylsilyl)- trifluoroacetamide (MSTFA) was added. The extracts were further incubated for 60 minutes at 60°C. PA-W and PA-M were analyzed using Perkin Elmer Clarus 600 Turbo Mass GC-MS (Perkin Elmer, USA) coupled to quadrupole type MS operated at 70 eV. The putative identification was carried out by comparing mass spectral library with National Institute of Standards and Technology (NIST) library (2008). 1 µL aliquot was injected into Elite 5MS (5% phenyl 95% dimethylpolysiloxane, 30 m × 0.25 mm ID × 0.25 µm) column. The flow rate of helium carrier gas was 1 mL/min. The initial oven temperature was set to 70°C and was raised at 1°C/min to 76°C and then at 6°C/min to 300°C. The total run time was 50.33 minutes. The full scan mode was acquired at a mass range of 50-500 m/z, with a solvent delay of 7 minutes. The injection and ion source (EI) temperature were adjusted to 250°C and 200°C respectively. Samples were injected in ratio mode (50:1).

3.5.4 Liquid chromatography mass spectrometry (LC-MS)

PA-W was subjected to LC-MS analyses. This is because out of five extracts available, PA-W showed the most positive result in antioxidant assays and xanthine oxidase inhibitory assay. The sample was prepared by diluting crude PA-W with HPLC grade water into 10 ppm concentration. The sample was then sent to INFRA Analytical Laboratory, University of Malaya, Kuala Lumpur for analysis. The phytochemical compounds of *P. amaryllifolius* were detected according to the method described by Kolniak-Ostek & Oszmiański (2015) with slight modification. The chromatographic separations of were carried out using LC-MS 6550 iFunnel Q-TOF System with C18 column (2.1 mm × 100 mm, 1.8 μm) at 30°C. 10 μL of sample was injected with the flow rate of 0.1 mL/min. The electrospray ionization (ESI) source was operating in negative mode. The mobile phase comprised of solvent A (0.1% formic acid, v/v) and solvent B (100% acetonitrile). A binary gradient of solvent A and solvent B was as follows: 99% solvent A (0–1 min), and then a linear gradient was used for 12 min, reducing solvent A to 0%; from 12.5 to 13.5 min, the gradient went back to the initial composition (99% solvent A), and then it was held constant to re-equilibrate the column.

3.6 Quantification of phenolic and flavonoid

3.6.1 Total phenolic content (TPC)

Folin-Ciocalteu's reagent was used to determine the total phenol content of *P. amaryllifolius* extracts using the method by Ablat *et al.* (2014) with a slight modification. Folin-Ciocalteu reagent was diluted 10-fold with distilled water. In a 96-well microplate, 20 μL extract was mixed with 100 μL of Folin-Ciocalteu reagent and incubated for five minutes. Next, 75 μL of sodium carbonate solution was added and the microplate was allowed to incubate in darkness for two hours at room temperature. After two hours, the absorbance was measured at 740 nm using a microplate reader (Tecan Sunrise, Austria). Gallic acid (concentration range: 0.0625–1.0 mM) was used as a standard to construct a linear regression line and the blank was water. The results were expressed as mg gallic acid equivalent (mg GAE)/g of dry extract.

3.6.2 Total flavonoid content (TFC)

Total flavonoid contents of *P. amaryllifolius* extracts were determined according to the method by Ablat *et al.* (2014). 50 μL of each extracts was added into a 96-well microplate along with 15 μL of 5% sodium nitrite solution and 70 μL of distilled water. After mixing the solution well, it was incubated for five minutes at room temperature. Next, 15 μL of 10% aluminum chloride solution was added and the solution was incubated for six minutes. Lastly, 100 μL of 1 M sodium hydroxide solution was added. The absorbance was measured at 510 nm with a microplate reader (Tecan Sunrise, Austria). Quercetin (concentration range: 0.2–1.0 mM) was used for standard calibration curve. The total flavonoid contents were evaluated according to the linear regression between standard solution and absorbance at 510 nm. The results were estimated as mg quercetin equivalent (mg QE)/g of dry extract.

3.7 Antioxidant activity

3.7.1 DPPH radical scavenging activity

The DPPH radical scavenging activity of *P. amaryllifolius* extracts was assessed according to the method depicted by Ablat *et al.* (2014) with several modifications. First, extracts (40 μL) at different concentrations (10–240 μg/mL) were added into a 96-well microplate. Then, 200 μL of 50 μM DPPH solution in ethanol was added and the mixture was instantly mixed and shaken. The microplate was allowed to incubate in the darkness for 15 minutes at room temperature. Absorbance was measured at 517 nm using a microplate reader (Tecan Sunrise, Austria). Ascorbic acid (concentration range: 10–240 μg/mL) was used as a standard and the control was ethanol. The percent inhibition of DPPH radical was calculated using the formula:

$$\text{DPPH radical scavenging activity (\%)} = \frac{A_{\text{control}} - A_{\text{sample or standard}}}{A_{\text{control}}} \times 100$$

The concentration of extracts needed in scavenging 50% of DPPH radical (IC_{50}) was determined from the graph plotted against the percentage of inhibition and contrasted with the standard. The entire tests were carried out in triplicate and the results were expressed in μg/mL.

3.7.2 Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was performed according to the method by Ablat *et al.* (2014) with a few modification. 20 μL of *P. amaryllifolius* extracts in ethanol were added into 96-well microplate. Then, 200 μL of FRAP reagent which is prepared fresh was added into each well containing the extracts. FRAP reagent was made by mixing 5 mL of 20 mM $FeCl_3$, 5 mL of 10 mM TPTZ in 40 mM HCl, and 50 mL of 0.3 M acetate buffer (pH

3.6). The mixture was incubated for eight minutes in to form the TPTZ-Fe^{2+} complex. Absorbance was recorded at 595 nm using a microplate reader (Tecan Sunrise, Austria). The standard was ferrous sulfate (concentration range: 0.125–1.0 mM) and the blank was ethanol. The FRAP value was determined from ferrous sulfate standard curve, and the results were expressed as mmol Fe^{2+}/g of dry extract.

3.7.3 Metal chelating activity

The ability of *P. amaryllifolius* extracts to chelate ferrous ion was measured according to the method described by Ablat *et al.* (2014) with slight modification. The test was initiated by mixing 100 μL of extracts of different concentrations (10–160 μg/mL), 10 μL of 2 mM $FeCl_2$, and 120 μL of distilled water together in a 96-well microplate. To start the reaction, 20 μL of 5 mM ferrozine was added into the mixture. After the incubation period of 20 minutes at room temperature, the absorbance was recorded at 562 nm. EDTA-Na_2 (concentration range: 5–160 μg/mL) was used as the standard and the control was ethanol (100 μL). The blank did not include the ferrozine but 20 μL of distilled water instead. Chelating activity was calculated relative to the control using the following formula:

$$\text{Ferrous ion chelating activity (\%)} = \frac{A_{control} - A_{sample\ or\ standard}}{A_{control}} \times 100$$

The concentration of extracts needed in chelating 50% of Fe^{2+} ion (IC_{50}) was determined from the graph plotted against the percentage of inhibition and contrasted with the standard. The entire tests were carried out in triplicate and the results were expressed in μg/mL.

3.7.4 Hydrogen peroxide scavenging activity

The *P. amaryllifolius* extracts scavenging ability of H_2O_2 molecules was assessed based on the method described by Khan *et al.* (2012) with a slight modification. First, 0.1 mL of extracts in ethanol (concentration range: 10–320 μg/mL) were added into the test tubes followed by the addition of 0.3 mL of 50 mM phosphate buffer (pH 7.4) to make the volume of 0.4 mL. After that, 0.6 mL of 2 mM hydrogen peroxide solution was added and the tubes were vortexed. The hydrogen peroxide solution was prepared in the 50 mM phosphate buffer (pH 7.4). After 10 minutes, the absorbance of hydrogen peroxide was then measured at 230 nm against the blank using UV-vis spectrophotometer-1700 (Shimadzu, Kyoto, Japan). Ascorbic acid (concentration range: 10–320 μg/mL) was used as a standard. The blank was 50 mM phosphate buffer without hydrogen peroxide. The percentage inhibition of hydrogen peroxide was calculated by the following equation:

$$\text{Hydrogen peroxide scavenging activity (\%)} = \frac{A_{\text{control}} - A_{\text{sample or standard}}}{A_{\text{control}}} \times 100$$

The concentration of extracts needed in chelating 50% of hydrogen peroxide (IC_{50}) was determined from the graph plotted against the percentage of inhibition and contrasted with the standard. The entire tests were carried out in triplicate and the results were expressed in μg/mL.

3.8 *In vitro* xanthine oxidase inhibitory activity

In vitro xanthine oxidase inhibitory activity was conducted on *P. amaryllifolius* extracts aerobically using the method by Azmi *et al.* (2012) with several modifications. 100 μL of extracts (concentration range: 6.25–100 μg/mL), 300 μL of 50 mM phosphate buffer (pH 7.5), and 100 μL of xanthine oxidase solution (0.1 units/mL in 50 mM phosphate buffer, pH 7.5) were added into a test tube. The enzyme solution was prepared fresh. The mixture was then incubated for 15 minutes at 37°C. The reaction was initiated after the addition of 200 μL of 0.15 mM xanthine solution in the same buffer, and incubated again for 30 minutes at 37°C. To stop the reaction, 200 μL of 0.5 M HCl was added. The absorbance was determined at 295 nm with UV-vis spectrophotometer-1700 (Shimadzu, Japan). Allopurinol (concentration range: 6.25–100 μg/mL) was the positive control. The preparation of blank was the same as the sample and standard. But the xanthine oxidase solution was substituted with the phosphate buffer. The control was 100 μL of DMSO. The inhibitory activity was calculated by the following equation:

$$\text{Xanthine oxidase inhibition (\%)} = \frac{A_{\text{control}} - A_{\text{sample or standard}}}{A_{\text{control}}} \times 100$$

The concentration of extracts needed to inhibit 50% of xanthine oxidase (IC_{50}) was determined from the graph plotted against the percentage of inhibition and contrasted with the standard. The entire tests were carried out in triplicate and the results were expressed in μg/mL.

3.9 *In vivo* xanthine oxidase inhibitory activity

3.9.1 Acute toxicity test

6–7 weeks old Spraque Dawley rats were kept in their cages in the animal house for two weeks for adaptation to the standard laboratory conditions. A total of six rats (three male, three female) were used to receive a single oral dose (2000 mg/kg) of PA-W prepared using distilled water as vehicle. The rats were kept over-night fasting prior to extract administration. The next day, the weights of the rats were recorded and the extract was administered via oral gavage. After extracts administration, food but not water was withheld for further 3–4 hours. Observation was performed to individual rat during the first 24 hours with extra attention to the first 4 hours. For the next 14 days, they were observed daily. The signs of toxicity to be detected include change in fur and skin, membranes of eyes and mucous, as well as respiratory, circulatory, nervous system, and somatomotor activity and behaviour pattern. Individual weights of the rats were recorded at the day of dosing, in weekly periods and at the day of execution. Mortality occurrence was determined over a period of two weeks. If the rats were found to be in a moribund state and exhibiting serious pain or undergoing signs of very bad distress, they should be humanely terminated. The number of dead and surviving animals determines the next dose to be administered (OECD 423).

3.9.2 Experimental animals

Healthy Sprague Dawley (SD) male rats (6–7 weeks old, 150–130 g) were used in hypouricemic activity experiment. The rats were purchased from University of Malaya Animal House. 36 male rats were housed in their cages for two weeks prior to dosing to allow for acclimatization to the laboratory condition according to OECD 423 guidelines. They were maintained under standard laboratory conditions throughout experiment with

the sequence of lighting being 12 hours light and 12 hours dark, under room temperature (37ºC). Conventional rat pellet diet and unlimited supply of tap water were used for feeding. The protocol used in this experiment fulfilled the ethical code sanctioned by the Faculty of Medicine Institutional Animal Care and Use Committee (FOM IACUC), University of Malaya (Ethic no: 2015-180908/IBS/R/NAAS).

3.9.3 Animal experimental design

(a) ***Preparation of the hyperuricemic rat model***

Hyperuricemic rat model was assigned to this experiment by intraperitoneal (i.p.) injection of the uricase inhibitor, oxonic acid potassium salt (280 mg/kg single dose) according to the method by Yonetani *et al.* (1980). The oxonic acid potassium salt solution was prepared by suspending it in 0.9% sodium chloride (NaCl) solution.

(b) ***Hypouricemic activity study***

Hypouricemic efficacy of PA-W was investigated using the method described by Umamaheswari *et al.* (2007) with a few modifications. In this experiment, 36 male rats were divided into six groups (n = 6). Group I was normal control group that received 0.5% CMC solution orally. Group II only received high dose PA-W (1000 mg/kg) via oral gavage. Group III was the hyperuricemic control that intraperitoneally administered with oxonic acid potassium salt (280 mg/kg, i.p.). Rats of group IV and V received high dose (1000 mg/kg) and low dose (500 mg/kg) of PA-W by oral gavage, respectively. Group VI was given oral treatment of allopurinol (10 mg/kg body weight). Table 3.1 summarized the experimental conditions. PA-W was dissolved in vehicle distilled water while allopurinol was dissolved in 0.5% CMC solution. One hour prior to extracts and allopurinol administration, hyperuricemic rats in groups III, IV, V, and VI were injected with oxonic acid potassium salt (280 mg/kg, i.p.) to elevate the serum uric acid level.

One hour after extract and allopurinol administration, small blood samples were collected by tail prick to measure serum urate level using Multi Sure Uric Acid Meter purchased from ApexBio (Taiwan) and the results were expressed as mg/dL. Right after the tail prick, all the rats were executed and whole blood samples were collected through intracardiac puncture where the blood was allowed to clot and serum separated. The xanthine oxidase activity of the collected serum was determined using Xanthine Oxidase Activity Assay Kit purchased from Sigma-Aldrich (St. Louis, MO, USA) and the results were expressed as milliunit/mL.

Table 3.1: Treatment groups

Group	Condition	Treatment
1	Normal rats	Control
2	Normal rats	High dose of PA-W [1000 mg/kg]
3	Hyperuricemic rats	Control
4	Hyperuricemic rats	High dose of PA-W [1000 mg/kg]
5	Hyperuricemic rats	Low dose of PA-W [500 mg/kg]
6	Hyperuricemic rats	Allopurinol [10 mg/kg]

3.10 Statistical analysis

The data values were represented as mean ± SEM (standard error of mean) for three replicates. Data were analyzed using one-way ANOVA followed by Dunnett's test for multiple group comparison. The statistical significance was at $P < 0.001$. The correlation between TPC, TFC, and antioxidant activities were assessed using Pearson correlation test ($P < 0.01$ and $P < 0.05$).

CHAPTER 4: RESULTS

4.1 Preparation of plant extracts

Dried *Pandanus amaryllifolius* Roxb. powder was extracted using five different solvents. *P. amaryllifolius* hexane extract (PA-H), petroleum ether extract (PA-PE), chloroform extract (PA-C), and methanol extract (PA-M) were obtained under reduced pressure via rotary evaporator whereas *P. amaryllifolius* water extract (PA-W) was obtained by freeze-drying method. The yield of the extracts is depicted in Table 4.1. Results obtained from this experiment showed that PA-W in dark brown gives the highest yield of 12.72% (3.18 g), followed by dark green coloured PA-M with the yield of 9.04% (2.26 g). From 25 g of dried weight of *P. amaryllifolius* powder, PA-H, PA-PE, and PA-C displayed 0.22% (0.054 g), 0.07% (0.017 g), and 0.36% (0.089 g) yield of extract respectively.

Table 4.1: Yields of *P. amaryllifolius* extracts in different solvents

Extracts	Dry weight (g)	Crude extract (g)	Yield crude extract (%)	Colour of crude extract
PA-H	25	0.054	0.22	Dark green
PA-PE	25	0.017	0.07	Dark green
PA-C	25	0.089	0.36	Dark green
PA-M	25	2.260	9.04	Dark green
PA-W	25	3.180	12.72	Dark brown

4.2 Identification of the phytochemical compounds

4.2.1 Thin layer chromatography (TLC)

The detection of phytochemical compounds was discovered by TLC analysis with different detection reagents. The first step in this analysis was developing appropriate mobile systems of solvents with different polarity can provide the best separation for each extracts. As a result, three solvent systems (hexane-acetone, 7:3; hexane-acetone, 8:2; chloroform-methanol, 1:9) were selected and used to separate these compounds. In this experiment, chlorophylls, flavonoids, phenols, terpenoids, and xanthophylls were detected in PA-H (Table 4.2). Chlorophylls, flavonoids, terpenoids, and xanthophylls were detected in PA-PE (Table 4.3). Chlorophylls, phenols, and terpenoids were detected in PA-C and PA-M (Table 4.4 and 4.5). Phenols and terpenoids were detected in PA-W (Table 4.6).

The colour results strongly suggest that terpenoids were present in all five extracts after the bands on TLC plates turned to purple colour when sprayed with Vanillin-H2SO4 reagent. The detection of chlorophylls was observed when bands turned into green colour in visible light. Chlorophylls were present in all extracts except for PA-W. Xanthophylls were found only in PA-H, PA-PE, and PA-C. Flavonoid turned bands into green colour after sprayed with anisaldehyde-sulphuric acid reagent. It was detected in PA-H and PA-PE but not detected in PA-C, PA-M, and PA-W possibly because they were present in very low concentrations. The TLC results showed that phenols were detected in all extracts except for PA-PE. The purple bands appeared after reacted with anisaldehyde-sulphuric acid reagent indicating the presence of phenolic compounds.

Table 4.2: TLC profile of PA-H in mobile system hexane:acetone (7:3)

Labelled compound	R_f value	Colour observation					Tentative ID of phytochemical
		Visible light	UV light	Detection with reagent			
				Dragendorff	Vanillin-H_2SO_4	ρ-anisaldehyde	
H1	0.44	yellow (++)	yellow (++)	-	-	-	xanthophyll
H2	0.51	green (+)	green (+)	-	-	purple (+)	phenol
H3	0.56	green (++)	green (++)	-	-	green (++)	flavonoid
H4	0.61	-	-	-	purple (+++)	-	terpenoid
H5	0.64	green (+++)	green (+++)	-	-	-	chlorophyll
H6	0.71	-	-	-	purple (+++)	-	terpenoid
H7	0.86	-	-	-	purple (++)	-	terpenoid
H8	0.91	-	-	-	purple (+++)	-	terpenoid

Indication of colour intensity: Weak (+), Medium (++), Strong (+++), No colour observed (-)

Table 4.3: TLC profile of PA-PE in mobile system hexane:acetone (8:2)

Labelled compound	R_f value	Colour observation					Tentative ID of phytochemical
		Visible light	UV light	Detection with reagent			
				Dragendorff	Vanillin-H_2SO_4	ρ-anisaldehyde	
P1	0.11	-	-	-	purple (+)	-	terpenoid
P2	0.17	yellow (++)	yellow (++)	-	-	-	xanthophyll
P3	0.26	green (++)	green (++)	-	-	-	cholrophyll
P4	0.31	green (+++)	green (+++)	-	-	-	chlorophyll
P5	0.37	-	-	-	-	green (+++)	flavonoid
P6	0.41	green (+++)	green (+++)	-	-	-	chlorophyll
P7	0.47	-	-	-	purple (+++)	-	terpenoid
P8	0.74	-	-	-	purple (+)	-	terpenoid

Indication of colour intensity: Weak (+), Medium (++), Strong (+++), No colour observed (-)

Table 4.4: TLC profile of PA-C in mobile system hexane:acetone (7:3)

Labelled compound	R_f value	Colour observation					Tentative ID of phytochemical
		Visible light	UV light	Detection with reagent			
				Dragendorff	Vanillin-H_2SO_4	ρ-anisaldehyde	
C1	0.09	green (+++)	green (+++)	-	-	-	chlorophyll
C2	0.19	green (+)	green (+)	-	-	-	chlorophyll
C3	0.28	-	-	-	purple (+)	-	terpenoid
C4	0.43	green (+)	green (+)	-	-	-	chlorophyll
C5	0.50	green (++)	green (++)	-	-	-	chlorophyll
C6	0.64	green (++)	green (++)	-	-	-	chlorophyll
C7	0.71	green (+++)	green (+++)	-	-	-	chlorophyll
C8	0.78	-	-	-	-	blue (+)	phenol
C9	0.89	-	-	-	-	blue (+)	phenol

Indication of colour intensity: Weak (+), Medium (++), Strong (+++), No colour observed (-)

Table 4.5: TLC profile of PA-M in mobile system hexane:acetone (7:3)

Labelled compound	R_f value	Colour observation					Tentative ID of phytochemical
		Visible light	UV light	Detection with reagent			
				Dragendorff	Vanillin-H_2SO_4	ρ-anisaldehyde	
M1	0.14	green (++)	green (++)	-	-	-	chlorophyll
M2	0.20	green (+)	green (+)	-	-	-	chlorophyll
M3	0.51	green (++)	green (++)	-	-	-	chlorophyll
M4	0.59	green (++)	green (++)	-	-	purple (+)	phenol
M5	0.64	green (++)	green (++)	-	-	-	chlorophyll
M6	0.71	green (+)	green (+)	-	purple (+)	-	terpenoid

Table 4.6: TLC profile of PA-W in mobile system chloroform:methanol (1:9)

Labelled compound	R_f value	Colour observation					Tentative ID of phytochemical
		Visible light	UV light	Detection with reagent			
				Dragendorff	Vanillin-H_2SO_4	ρ-anisaldehyde	
W1	0.62	yellow (+)	purple (++)	-	-	purple (+)	phenol
W2	0.87	-	purple (++)	-	purple (++)	-	terpenoid

Indication of colour intensity: Weak (+), Medium (++), Strong (+++), No colour observed (-)

4.2.2 Gas chromatography mass spectrometry (GC-MS)

In this study, phytochemical compounds present in PA-W and PA-M were identified using gas chromatography mass spectrometry. The analysis led to the identification of 31 compounds in PA-W and 30 compounds in PA-M. In PA-W, organic acids, fatty acid, amino acid, amino alcohol, pyrimidine, sugar, and polyol were present. In PA-M, organic acids, amino alcohol, amino sugar, sugar, and polyol were present. Based on the chromatogram profile of GC-MS reflected in Figure 4.1, the peaks were marked with its retention time. The peak with the highest intensity comes at 27.55 min and 27.10 min for PA-W and PA-M, representing D-Glucose and D-Fuctose respectively. In both extracts, several identical compounds were detected. At 15.19 min and 15.16 min, glycerol was identified in PA-W and PA-M, as well as butanedioic acid at 16.53 min and 16.48 min, 1,4-butanediol at 20.51 min and 20.50 min, L-(-)-Arabitol at 24.51 min and 24.49 min, and 1-Cyclohexene-1-carboxylic acid at 26.23 min and 26.20 min, respectively. Glucopyranose was detected in both extract at 33.69 min. Cinnamic acid and benzoic acid were the phenols detected in PA-W at 28.34 min and 28.51 min respectively. In PA-M, benzoic acid was detected at 26.30 min. Compounds detected were summarized in Table 4.7 and Table 4.8.

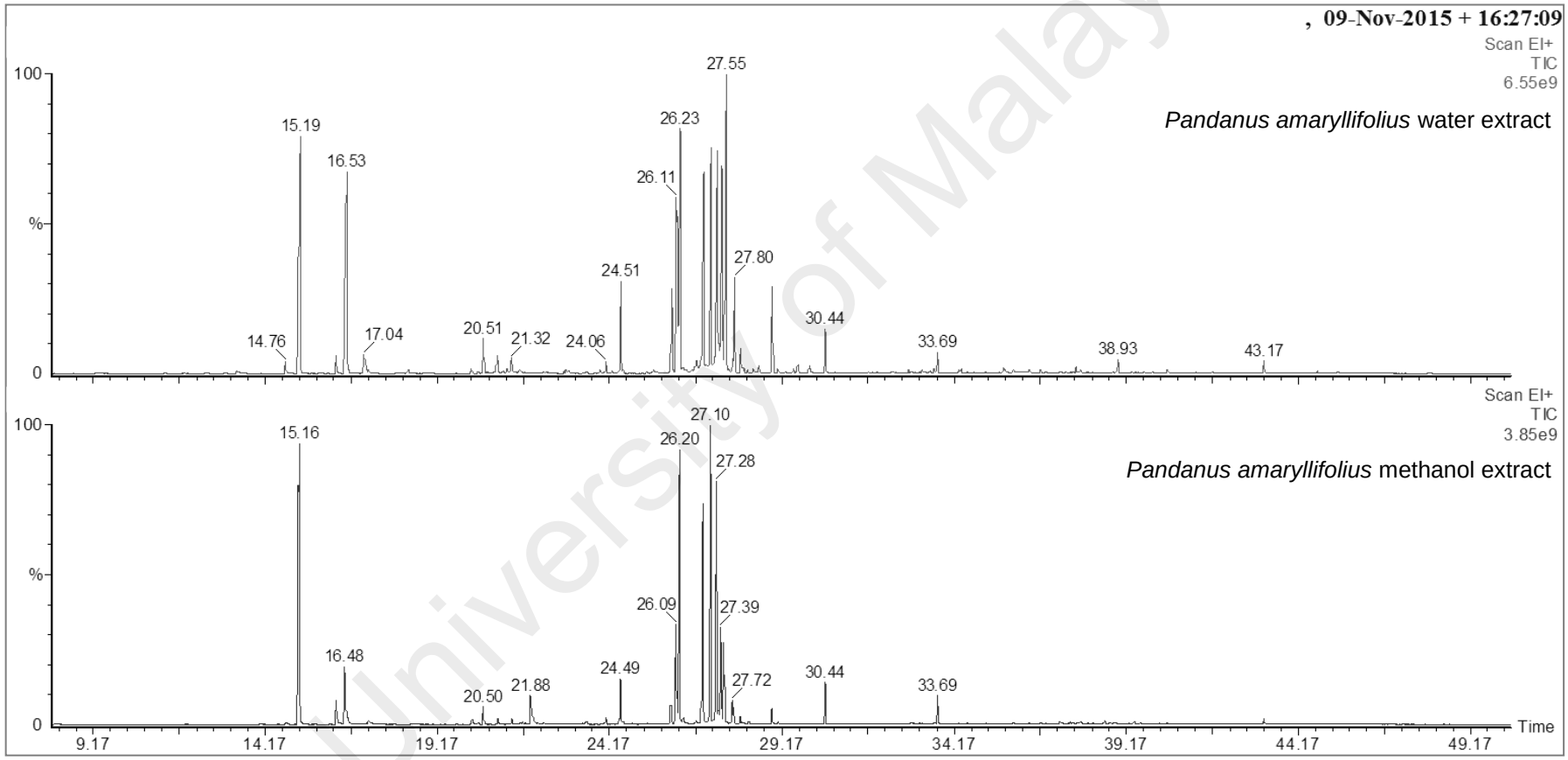

Figure 4.1: GC-MS chromatogram profile of PA-W and PA-M

Table 4.7: Phytochemical compounds in PA-W detected by using GC-MS

No	RT value	Compound ID	Group
1	9.36	L-Valine	Amino acid
2	14.76	Silanamine	Amino alcohol
3	15.19	Glycerol	Polyol
4	16.53	Butanedioic acid	Organic acid
5	17.04	Butanedioic acid	Organic acid
6	17.17	Fumaric acid	Organic acid
7	18.32	Mesaconic acid	Organic acid
8	20.15	Malic acid	Organic acid
9	20.51	1,4-Butanediol	Polyol
10	20.90	Butanoic acid	Organic acid
11	24.06	Arabinitol	Polyol
12	24.51	L-(-)-Arabitol	Polyol
13	25.56	Ribonic acid	Organic acid
14	25.97	D-Xylofuranose	Sugar
15	26.11	D-(-)-Fructose	Sugar
16	26.23	1-Cyclohexene-1-carboxylic acid	Organic acid
17	27.31	D-Fructose	Sugar
18	27.43	Ribitol	Polyol
19	27.55	D-Glucose	Sugar
20	27.75	D-Galactose	Sugar
21	27.80	D-Glucose	Sugar
22	28.34	Cinnamic acid	Organic acid
23	28.51	Benzoic acid	Organic acid
24	29.05	á-D-Galactofuranose	Sugar
25	29.64	Mannose	Sugar
26	29.96	Hexadecanoic acid	Fatty acid
27	30.44	D-(+)-Xylose	Sugar
28	33.69	Glucopyranose	Sugar
29	35.61	Uridine	Pyrimidine
30	38.93	Thymol-á-d-glucopyranoside	Organic acid
31	43.17	Glycoside	Sugar

Table 4.8: Phytochemical compounds in PA-M detected by using GC-MS

No	RT value	Compound ID	Group
1	8.02	Propanoic acid	Organic acid
2	14.77	Silanamine	Amino alcohol
3	15.16	Glycerol	Polyol
4	16.48	Butanedioic acid	Organic acid
5	17.16	Fumaric acid	Organic acid
6	18.43	Mesaconic acid	Organic acid
7	20.18	Malic acid	Organic acid
8	20.50	1,4-Butanediol	Polyol
9	20.91	Butanoic acid	Organic acid
10	23.50	D-Ribose	Sugar
11	24.05	Arabinitol	Polyol
12	24.49	L-(-)-Arabitol	Polyol
13	25.95	D-Ribofuranose	Sugar
14	26.09	D-Fructose	Sugar
15	26.20	1-Cyclohexene-1-carboxylic acid	Organic acid
16	26.30	Benzoic acid	Organic acid
17	27.10	D-Fructose	Sugar
18	27.28	D-Fructose	Sugar
19	27.39	D-Galactose	Sugar
20	27.49	DGlucose	Sugar
21	27.72	D-Galactose	Sugar
22	27.77	D-Glucose	Sugar
23	28.02	D-Glucosamine	Amino Sugar
24	29.06	Ribonic acid	Organic acid
25	30.44	Inosose-2	Sugar
26	33.61	Glycoside	Sugar
27	33.69	Glucopyranose	Sugar
28	34.54	D-Xylopyranose	Sugar
29	38.54	à-D-Galactopyranosiduronic acid	Organic acid
30	43.17	à-D-Galactopyranose	Sugar

4.2.3 Liquid chromatography mass spectrometry (LC-MS)

Phytochemical compounds present in PA-W were identified using liquid chromatography mass spectrometry. Three groups which are organic acids, ketone, and ester were present in PA-W. According to the chromatogram profile of LC-MS reflected in Figure 4.2, all four compounds were detected around 3 to 4 min of retention time. At 3.02 min and 3.05 min, organic acids, L-galactonate and glucuronic acid were detected respectively. At 3.32 min, 2D-5-O-Methyl-2,3,5/4,6-pentahydroxycyclohexanone was detected. At 3.97 min, 4-Methyl-3-oxoadipate was detected. The identified compounds were listed in Table 4.9.

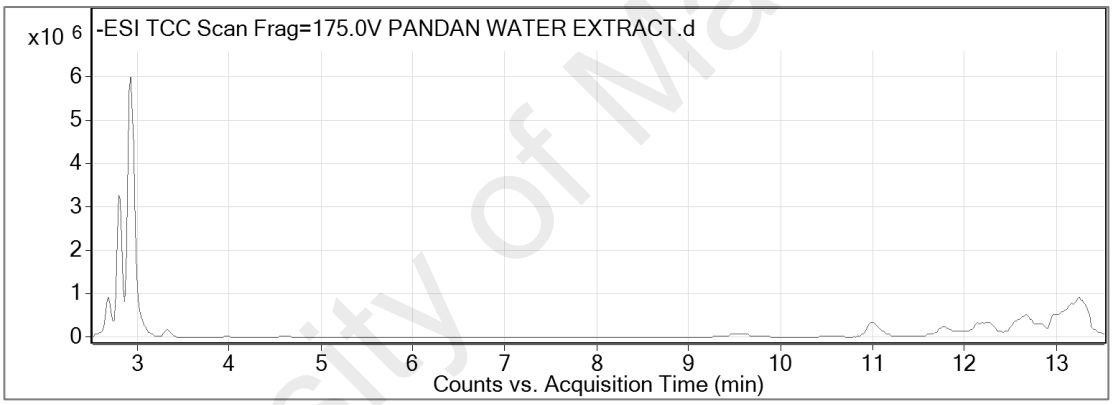

Figure 4.2: LC-MS chromatogram profile of PA-W

Table 4.9: Phytochemical compounds in PA-W detected by using LC-MS

No	RT value	Compound ID	Group
1	3.02	L-Galactonate	Organic acid
2	3.05	Glucuronic acid	Organic acid
3	3.32	2D-5-O-Methyl-2,3,5/4,6-pentahydroxycyclohexanone	Ketone
4	3.97	4-Methyl-3-oxoadipate	Ester

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4.3 Quantification of phenolic and flavonoid

4.3.1 Total phenolic content (TPC)

Total phenol content was expressed as gallic acid equivalent (mg GAE)/g of dry extract by comparing to a gallic acid standard curve (y=1.024x+0.21, R^2=0.918). The result on the phenolic contents of *P. amaryllifolius* extracts obtained from the extraction by different solvents is depicted in Table 4.10. PA-W (12.88 ± 0.43 mg GAE/g dry extract) showed higher phenolic content as compared to PA-M (11.98 ± 0.40 mg GAE/g dry extract). It was then followed by PA-C (6.42 ± 0.49 mg GAE/g dry extract), PA-PE (3.65 ± 0.26 mg GAE/g dry extract), and finally PA-H (2.32 ± 0.27 mg GAE/g dry extract). The results of TPC showed that with increasing polarity of the solvent, the amount of phenolic compounds increased.

4.3.2 Total flavonoid content (TFC)

Total flavonoid content was expressed as quercetin equivalent (mg QE)/g of dry extract by referring to a standard curve of quercetin (y=0.283x+0.042, R^2=0.957). The result on the flavonoid contents *P. amaryllifolius* extracts obtained from the extraction by different solvents is depicted in Table 4.10. PA-PE (15.02 ± 0.58 mg QE/g dry extract) possessed the highest flavonoid content followed by PA-H (11.66 ± 1.01 mg QE/g dry extract), PA-C (9.19 ± 1.53 mg QE/g dry extract), PA-M (9.07 ± 0.36 mg QE/g dry extract), and lastly PA-W (7.66 ± 1.10 mg QE/g dry extract). TFC was found higher in solvent with low polarity like PA-PE and PA-H. This was supported by TLC result as flavonoid was detected in PA-H (R_f value = 0.56) and PA-PE (R_f value = 0.37).

Table 4.10: TPC and TFC of *P. amaryllifolius* extracts

Extracts	TPC (mg GAE/g dry extract)	TFC (mg QE/g dry extract)
PA-H	2.32 ± 0.27	11.66 ± 1.01
PA-PE	3.65 ± 0.26	15.02 ± 0.58
PA-C	6.42 ± 0.49	9.19 ± 1.53
PA-M	11.98 ± 0.40	9.07 ± 0.36
PA-W	12.88 ± 0.43	7.66 ± 1.10

Values are expressed as mean ± SEM (n = 3)

4.4 Antioxidant activity

The antioxidant activity determination of *P. amaryllifolius* extracts was conducted in four assays including DPPH radical scavenging activity, ferric reducing antioxidant power (FRAP) assay, metal chelating activity, and hydrogen peroxide scavenging activity.

4.4.1 DPPH radical scavenging activity

DPPH assay showed that antioxidant activity of *P. amaryllifolius* extracts was affected by the nature of solvent used. The result portrayed in Figure 4.3 indicates that IC_{50} was not achieved in all extracts when the same concentrations as the standard were applied. However, each extract possessed steady increase in the DPPH inhibition activity in the range of 10–240 μg/mL. Based on the graph, PA-W showed the highest DPPH inhibition compared to others. This followed by PA-M, PA-C, PA-PE, and PA-H. The IC_{50} value of ascorbic acid was 32.21 ± 1.54 μg/mL (Table 4.11). The mechanism of this assay involves the formation of stable DPPH-H non radical form with the help of hydrogen donating antioxidants present in the extracts. The higher the amount of antioxidants in a sample, the higher the DPPH radical scavenging activity. The decrease in absorbance was detected at 517 nm. In this assay, the colour of the

reaction mixture turned from violet to pale yellow indicating positive results of scavenged DPPH radicals.

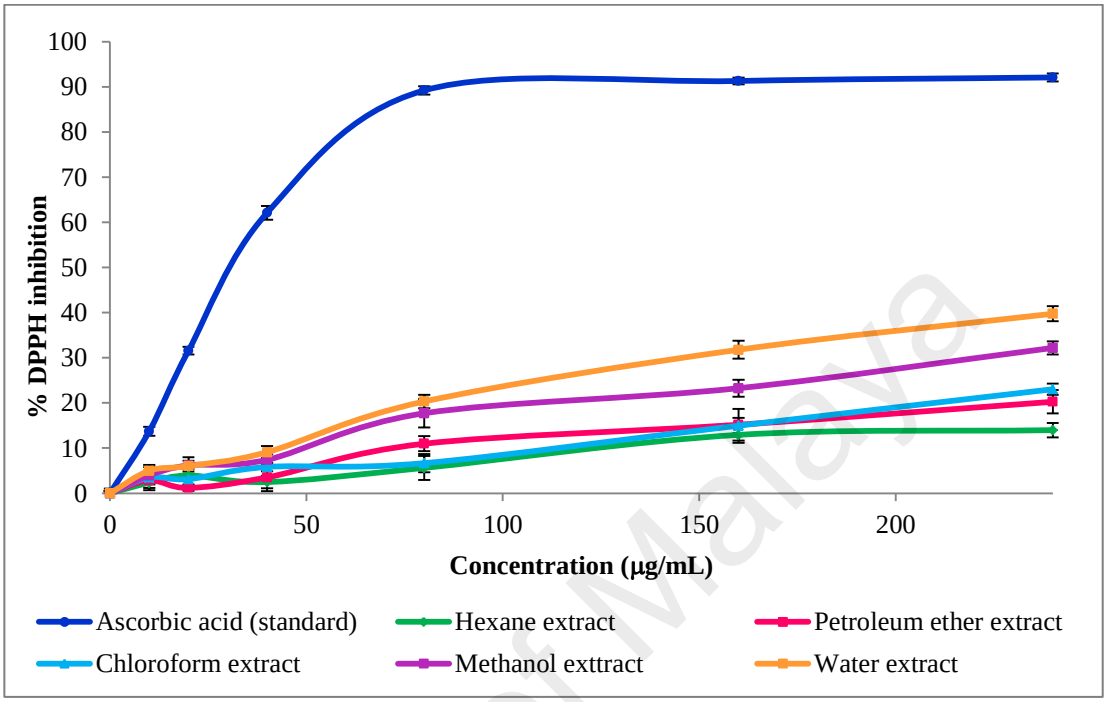

Figure 4.3: Effect of *P. amaryllifolius* extracts on DPPH radical scavenging activity

4.4.2 Ferric reducing antioxidant power (FRAP) assay

The result of FRAP assay is expressed as FRAP value (mmol Fe^{2+}/g of dry extract). Based on Table 4.11 the FRAP value of PA-H, PA-PE, PA-C, PA-M, and PA-W are 21.21 ± 2.17, 28.64 ± 1.86, 35.91 ± 2.23, 64.39 ± 2.79, and 54.55 ± 1.48 mmol Fe^{2+}/g of dry extract respectively. The reaction involves the formation of blue colour Fe^{2+}-TPTZ form in the extracts after the antioxidants react with Fe^{3+}-TPTZ complex. The highest FRAP value was detected in PA-M with 64.39 ± 2.79 mmol Fe^{2+}/g of dry extract. The reducing ability of PA-H was the lowest as it only showed 21.21 ± 2.17 mmol Fe^{2+}/g of dry extract. The reducing power was detected higher in polar solvent as compared to non polar solvent.

4.4.3 Metal chelating activity

In metal chelating assay, each extract was unable to achieve IC_{50} using the same concentration as EDTA-Na_2 standard. However, PA-W portrayed the strongest effectiveness in inhibiting the formation of Fe^{2+}-ferrozine complex followed by PA-H, PA-M, PA-PE, and lastly PA-C (Figure 4.4). EDTA-Na_2 achieved IC_{50} value of 11.23 ± 2.13 µg/mL (Table 4.11). IC_{50} value is defined as the concentration of test compound required to achieve 50% maximal inhibition. Even without the capability to obtain IC_{50}, each extract did possess the ability to chelate metal ferrous ions within the concentration range of 5–160 µg/mL. The decrease in absorbance was detected at 562 nm. From the results, the extracts showed metal chelating capability in dose dependent manner.

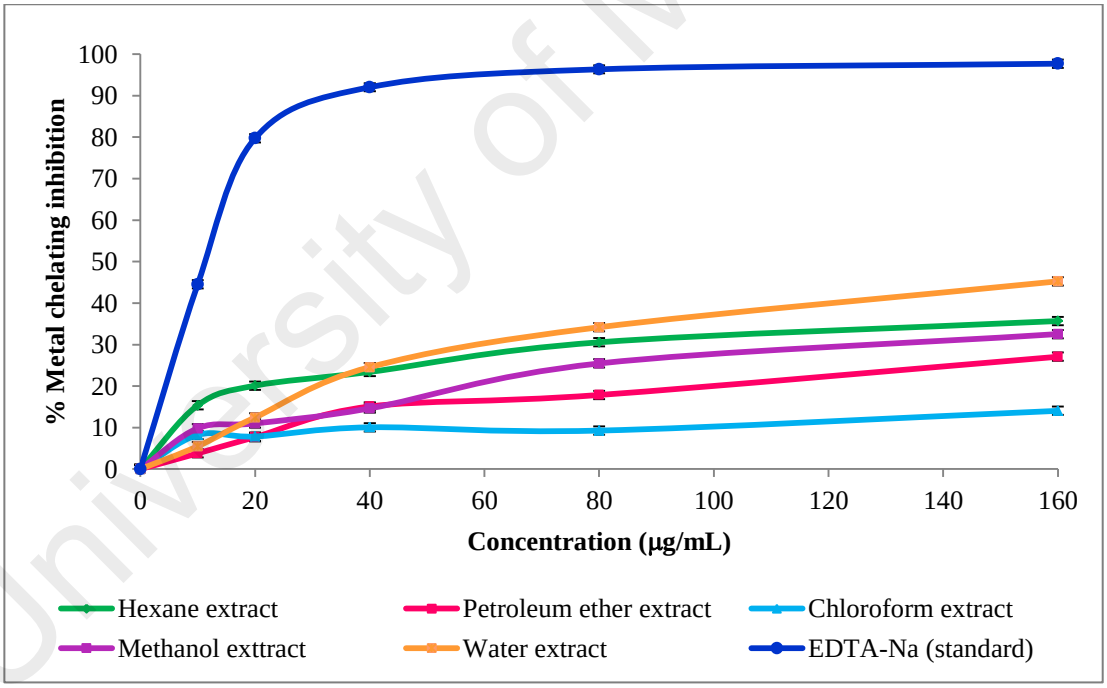

Figure 4.4: Effect of *P. amaryllifolius* extracts on metal chelating activity

4.4.4 Hydrogen peroxide scavenging activity

Hydrogen peroxide assay revealed that only PA-W and PA-M possess H_2O_2 scavenging capability among four *P. amaryllifolius* extracts tested (Figure 4.5). Both extracts possessed hydrogen peroxide scavenging capability despite the fact that IC_{50} was not achieved in the concentration range of 10–320 µg/mL. PA-H and PA-C exhibited negative scavenging activity. PA-PE was not included since the yield of the crude extract was insufficient to proceed with this test. Ascorbic acid standard exhibited inhibition with IC_{50} value of 161.92 ± 2.03 µg/mL (Table 4.11).

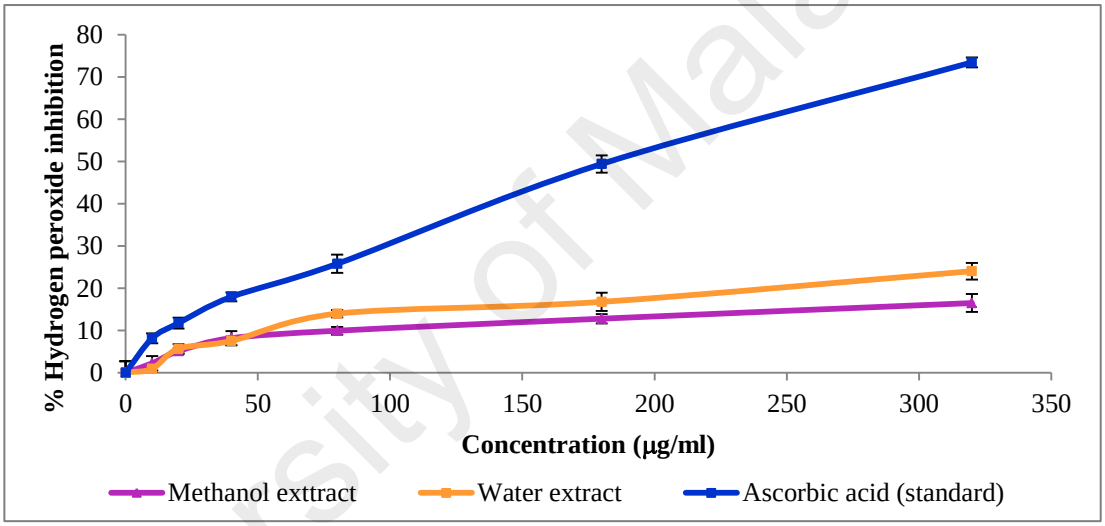

Figure 4.5: Effect of *P. amaryllifolius* extracts on hydrogen peroxide scavenging activity

Table 4.11: Antioxidant activities of *P. amaryllifolius* extracts

Extracts	DPPH (IC_{50} µg/mL)	FRAP (mmol Fe^{2+}/g of dry extract)	Metal chelating (IC_{50} µg/mL)	Hydrogen Peroxide (IC_{50} µg/mL)
PA-H	>240	21.21 ± 2.17	>160	-
PA-PE	>240	28.64 ± 1.86	>160	-
PA-C	>240	35.91 ± 2.23	>160	-
PA-M	>240	64.39 ± 2.79	>160	>320
PA-W	>240	54.55 ± 1.48	>160	>320
Standard	32.21 ± 1.54	-	11.23 ± 2.13	161.92 ± 2.03

Values are expressed as mean ± SEM (n = 3)

4.5 *In vitro* xanthine oxidase inhibitory activity

The ability of *P. amaryllifolius* extracts in inhibiting xanthine oxidase activity showed that only PA-W, PA-M, and PA-H possessed xanthine oxidase inhibition ability with PA-W having the highest activity (>100 µg/mL) (Figure 4.6). IC_{50} however was not achieved in all three extracts except for allopurinol standard (IC_{50} = 9.30 ± 1.17 µg/mL) (Table 4.12). Since PA-W exhibited the most expressive outcome compared to other extracts, it was chosen to be administered on rats for *in vivo* xanthine oxidase activity.

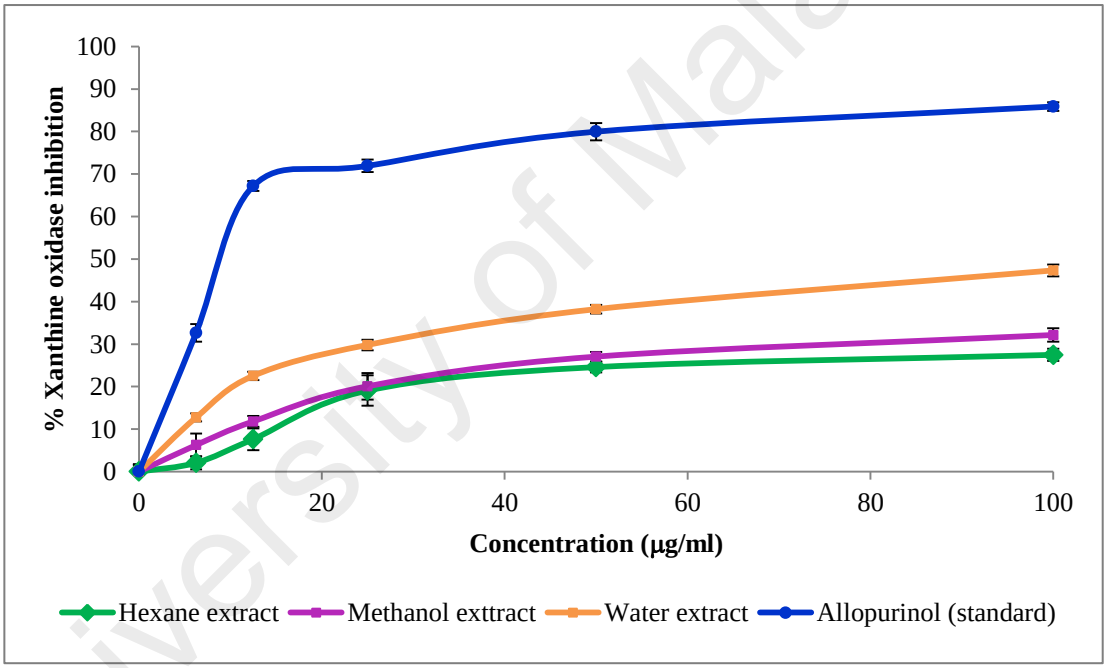

Figure 4.6: Effect of *P. amaryllifolius* extracts on *in vitro* xanthine oxidase inhibitory activity

Table 4.12: *In vitro* xanthine oxidase inhibitory activity of *P. amaryllifolius* extracts

Extracts	IC_{50} (µg/mL)
PA-H	>100
PA-M	>100
PA-W	>100
Standard (Allopurinol)	9.30 ± 1.17

Values are expressed as mean ± SEM (n = 3)

4.6 Correlation analysis

From the Pearson correlation tests summarized in Table 4.12, TPC of *P. amaryllifolius* extracts showed positive significant correlation with DPPH radical scavenging activity (r = 0.972) and FRAP assay (r = 0.964) at 0.01 level. There was also positive significant correlation between TPC and hydrogen peroxide scavenging activity (r = 0.898) at 0.05 level. TPC demonstrated no correlation with metal chelating activity (r = 0.382) and *in vitro* xanthine oxidase inhibitory activity (r = 0.809). On the other hand, TFC showed negative correlation with all assays.

Table 4.13: Correlation analysis

Assays	Correlation coefficients (r)	
	TPC	**TFC**
TPC	-	-0.786
TFC	-0.786	-
DPPH radical scavenging activity	0.972**	-0.761
FRAP assay	0.964**	-0.689
Metal chelating activity	0.382	-0.350
Hydrogen peroxide scavenging activity	0.898*	-0.691
In vitro xanthine oxidase inhibitory activity	0.809	-0.684

** Correlation is significant at the 0.01 level (2-tailed)
* Correlation is significant at the 0.05 level (2-tailed)

4.7 *In vivo* xanthine oxidase inhibitory activity

4.7.1 Acute toxicity test

Spraque Dawley rats of both sexes were fasted over-night before the administration of PA-W by oral gavage. After the study period of 14 days, it was found that the rats were safe at the dose of 2000 mg/kg. The rats showed normal behaviour pattern throughout the test. There were no signs of toxicity detected and zero occurrence of mortality in all rats. Based on this result, 1000 mg/kg (high dose) and 500 mg/kg (low dose) were selected for subsequent hypouricemic study.

4.7.2 Serum uric acid level

Administration of oxonic acid potassium salt to rats (280 mg/kg body weight, i.p.) caused a significant ($P < 0.001$) increase in serum uric acid level of Group 3 rats (16.57 ± 2.98 mg/dL) when compared to normal control rats (Group 1) after two hours (Table 4.13). Rats in Group 4 and 5 that received 1000 mg/kg and 500 mg/kg dose of PA-W portrayed significant ($P < 0.001$) decrease in the serum urate level up to 2.55 ± 2.23 mg/dL and 6.08 ± 1.00 mg/dL, when compared to hyperuricemic control, respectively. This showed that PA-W possesses a dose-dependently hypouricemic action in hyperuricemic rats. Group 6 rats treated with allopurinol also significantly reduced the serum uric acid level in hyperuricemic rats and showed practically similar serum urate level as normal control group (Figure 4.7).

Table 4.14: Serum uric acid level in hypouricemic study

Group	Condition	Treatment	Serum uric acid level (mg/dL)
1	Normal rats	Control	1.00 ± 0.00
2	Normal rats	High dose of PA-W [1000 mg/kg]	1.80 ± 1.13
3	Hyperuricemic rats	Control	16.57 ± 2.98[a]
4	Hyperuricemic rats	High dose of PA-W [1000 mg/kg]	2.55 ± 2.23[b]
5	Hyperuricemic rats	Low dose of PA-W [500 mg/kg]	6.08 ± 1.00[b]
6	Hyperuricemic rats	Allopurinol [10 mg/kg]	1.72 ± 1.01[b]

Values are expressed as mean ± SEM (n = 6).
[a] $P < 0.001$ significant when compared to normal control
[b] $P < 0.001$ significant when compared to hyperuricemic control

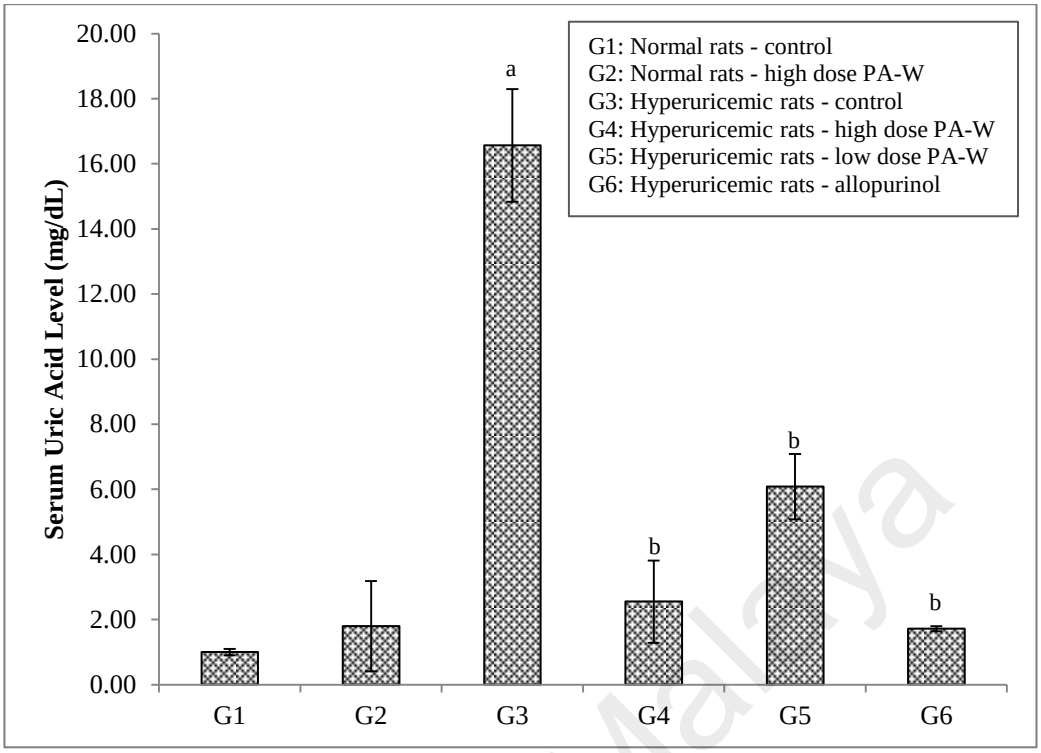

Figure 4.7: Serum uric acid level in hypouricemic study. [a] $P < 0.001$ indicates significant difference when compared to normal control. [b] $P < 0.001$ indicates significant difference when compared to hyperuricemic control.

4.7.3 Xanthine oxidase activity

After whole blood samples were collected and serum separated, the serum was further analyzed using Xanthine Oxidase Activity Assay Kit (Sigma-Aldrich, USA). The results were expressed as nmole/min/mL = mu/mL, where one milliunit (mU) of XO is defined as the amount of enzyme that catalyzes the oxidation of xanthine yielding 1.0 mmole of uric acid and hydrogen peroxide per minute at 25°C. Results obtained from this analysis showed that Group 3 possessed the highest XO activity followed by Group 5 and Group 4. Group 1, Group 2 and Group 6 exhibited low XO activity (Figure 4.8). When compared to normal control group (1.77 ± 0.43 mu/mL), serum of hyperuricemic rats in Group 3 displayed significant ($P < 0.001$) increase in XO activity with 10.72 ± 1.17 mu/mL (Table 4.14) because of the oxonic acid potassium salt. XO

activity in the serum of hyperuricemic rats treated with PA-W (high and low dose) and allopurinol were significantly reduced when compared to hyperuricemic control (Group 3) with 3.84 ± 0.68 mu/mL, 6.35 ± 0.87 mu/mL, and 1.06 ± 0.21 mu/mL respectively.

Table 4.15: Serum xanthine oxidase (XO) activity in hypouricemic study

Group	Condition	Treatment	XO activity (mu/mL)
1	Normal rats	Control	1.77 ± 0.43
2	Normal rats	High dose of PA-W [1000 mg/kg]	1.46 ± 0.41
3	Hyperuricemic rats	Control	10.72 ± 1.17[a]
4	Hyperuricemic rats	High dose of PA-W [1000 mg/kg]	3.84 ± 0.68[b]
5	Hyperuricemic rats	Low dose of PA-W [500 mg/kg]	6.35 ± 0.87[b]
6	Hyperuricemic rats	Allopurinol [10 mg/kg]	1.06 ± 0.21[b]

Values are expressed as mean ± SEM (n = 6).
[a] $P < 0.001$ significant when compared to normal control
[b] $P < 0.001$ significant when compared to hyperuricemic control

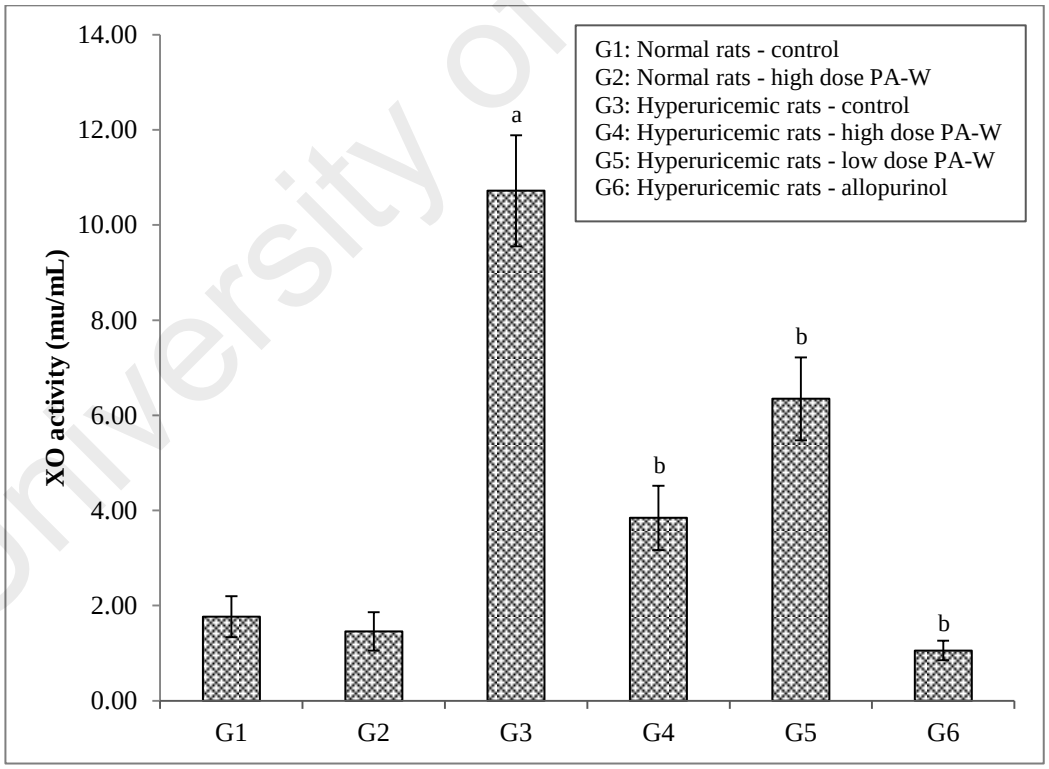

Figure 4.8: Serum xanthine oxidase (XO) activity in hypouricemic study. [a] $P < 0.001$ indicates significant difference when compared to normal control. [b] $P < 0.001$ indicates significant difference when compared to hyperuricemic control.

CHAPTER 5: DISCUSSION

5.1 Preparation of plant extracts

In this study, *Pandanus amaryllifolius* Roxb. plant underwent grounding process to become a powder using a blender. It was extracted using five different solvents, in order of increasing polarity which are hexane, petroleum ether, chloroform, methanol, and distilled water. The extraction process in 40°C water bath appeared to increase extract yield. Study by Akowuah *et al.* (2009) stated 40°C as the optimum temperature in the extraction of phenolic compounds and at higher temperature, low level of phenols was detected. This is possibly because increased temperature leads to the degradation of phenolic compounds. The moderate heat aids in concentrating the chemical compounds and amplifying the rate of extraction process. Out of these five solvents, water and methanol possessed an outstanding yield of crude extracts with 3.18 g and 2.26 g respectively. According to Ablat *et al.* (2014) polar solvents separate amino acids, hydrophilic molecules, and proteins whereas non polar solvents separate lipids. Havsteen (2002) stated that polar solvents can increase the efficiency of extraction process.

5.2 Identification of the phytochemical compounds

Plant based phytochemicals possess noteworthy impact in preventing numerous chronic diseases, convey beneficial outcome and perform crucial role in metabolic defects (Upadhyay & Dixit, 2015). The first step in detecting chemical constituents of *P. amaryllifolius* extracts in this research is using thin layer chromatography (TLC). The chemical compounds were separated and determined using spray reagents. The change

of colour on TLC plates confirmed the presence of specific compound. Different number of bands suggested that the nature of solvents used during extraction influences the kind of chemicals to be extracted.

Gas chromatography mass spectrometry (GC-MS) was conducted to discover phytochemical compounds present in PA-W and PA-M. Wong *et al.* (2015) stated that GC-MS is the recommended method to be used in analyzing metabolites particularly in plant extracts, quantitatively and qualitatively. PA-W and PA-M were chosen because both extracts showed strong performance in antioxidant studies, *in vitro* xanthine oxidase inhibitory assay, and generated higher yield of crude extracts compared to three other extracts which are PA-H, PA-PE, and PA-C. Furthermore, more extracts are needed to proceed with subsequent animal study. In addition, study by Płotka-Wasylka *et al.* (2017) highlighted the significance of avoiding the use of organic solvents during extraction as it is harmful, consumes large amount of resources, and able to produce hazardous and poisonous waste. Therefore, these were the reasons to why only PA-M and PA-W were selected to detect the presence of phytochemicals in *P. amaryllifolius* using GC-MS. Based on the analysis, PA-W showed the total presence of 31 compounds while PA-M with 30 compounds. The phytochemical compounds range from organic acid, fatty acid, amino acid, amino alcohol, pyrimidine, sugar, and polyol in PA-W. On the other hand, there were organic acid, amino alcohol, amino sugar, sugar, and polyol present in PA-M. In PA-W, two phenols were detected which are benzoic acid and cinnamic acid. In PA-M, one pheolic compound was detected which is benzoic acid. According to Azizan *et al.* (2012) GC-MS involves derivatization process that makes metabolites to be chemically volatile. Regular derivatization reagent, trimethylsilyl- (TMS) employed in this experiment gives a broad range of chemical detection. Chen *et al.* (2010) claimed that TMS is helpful for the detection of polyols, sugars and compounds that contain sulfur.

Liquid chromatography mass spectrometry (LC-MS) was carried out to qualitatively detect phytochemical compounds in PA-W. The analysis was conducted in negative ionization mode. Several studies used negative mode for LC-MS detection of phenolic compounds (Chen *et al.,* 2012; Zhu *et al.,* 2012; Bataglion *et al.,* 2014; Junqueira-Gonçalves *et al.,* 2015; Oszmiański *et al.,* 2015). Sun *et al.* (2013) stated that negative ionization mode showed better sensitivity and reproducibility than positive mode in full scan mass spectra. Study by Kolniak-Ostek & Oszmiański (2015) showed that major phenolic compounds such as flavonols, flavones, phenolic acids, hydrochalcones, catechins and procyanidins were putatively detected in negative ionization mode. Out of 152 compounds detected, the negative ionization mode used in the identification process revealed four known compounds which are L-galactonate, glucuronic acid, 2D-5-O-methyl-2,3,5/4,6-pentahydroxycyclohexanone, and 4-methyl-3-oxoadipate. These four compounds were not the same as the ones reported in previous phytochemical studies of *P. amaryllifolius.* For instances, 2AP, the major compound that impart pleasant smell in *P. amaryllifolius* (Yahya *et al.,* 2010) and phenolics such as phenolic acids (gallic acid) and flavonoids (catechin, kaempferol and naringin) (Ghasemzadeh & Jaafar, 2013).

There are several phytochemical compounds that perform well as xanthine oxidase inhibitor. Wan *et al.* (2016) showed that verbascoside, a polyphenol, significantly inhibited xanthine oxidase activity and reduced uric acid in hyperuricemic model. Other phenolics that showed potent inhibitory effect of xanthine oxidase are flavone eupatilin (Lin *et al.,* 2014) as well as phenolic acids (gallic acid, chlorogenic acid, caffeic acid, and ellagic acid) and flavonoids (catechin, rutin, luteolin, quercetin, epicatechin, and apigenin) (Irondi *et al.,* 2016). From the results obtained through GC-MS and LC-MS, it was revealed that most of the compounds detected in both extracts were comparatively similar. The organic acids which mainly comprised of carboxylic acids as well as sugars were found abundant in both extracts.

5.3 Quantification of phenolic and flavonoid

Total phenolic content (TPC) and total flavonoid content (TFC) of *P. amaryllifolius* extracts were determined to quantify total phenolics and flavonoids available in each extract. According to Karimi *et al.* (2011) phenolics are prominent secondary metabolites that exhibits extensive pharmacological actions while flavonoids are crucial secondary metabolites that possesses a number of bioactive compounds in plants. TPC was expressed as gallic acid equivalent by referring to a standard curve of gallic acid ($y=1.024x+0.21$, $R^2=0.918$). On the other hand, TFC was expressed as quercetin equivalent using standard curve of quercetin ($y=0.283x+0.042$, $R^2=0.957$). PA-W possessed the highest TPC while the highest TFC is represented by PA-PE. Based on Pearson correlation test carried out, TPC and TFC demonstrated negative correlation. Both of these experiments did not reflect on one another. This is because the amount of flavonoids was not in agreement with the amount of phenols represented by each extract. TFC was found higher in extracts of non polar solvents. On the other hand, TPC was found higher in extracts of polar solvents. This supports the study by Ablat *et al.* (2014) as they found that the amount of TPC increased as the polarity of the solvent. According to Kim *et al.* (2012) the extractants used in the extraction process had an impact on the TPC of extracts. Based on the detection of phytochemical compounds using GC-MS, the highest phenolic content in PA-W may be contributed by the presence of benzoic acid and cinnamic acid. Meanwhile, the present of benzoic acid may lead to the second highest phenolic content rank represented by PA-M.

5.4 Antioxidant activity

According to Sáyago-Ayerdi *et al.* (2007) more than one method is required to assess antioxidant potential. Determination of antioxidant activity was carried out using DPPH radical scavenging activity, ferric reducing antioxidant power (FRAP) assay, metal chelating activity, and hydrogen peroxide scavenging activity. According to Oskoueian *et al.* (2011) antioxidants aid in obstructing oxidative damage and phenolics can exhibit higher activity than vitamins, but its activity relies on their chemical structure and number of hydroxyl groups. Maltas *et al.* (2011) stated that high amount of phytochemicals such as phenolics, flavonoids, terpenoids and other result in high antioxidant activity. In this study, the antioxidant activities of *P. amaryllifolius* extracts were affected by the nature of solvent used.

Bajpai *et al.* (2017) claimed that DPPH scavenging activity is performed extensively by researchers to estimate antioxidant capabilities within short period of time than other assays. In this study, DPPH assay revealed PA-W as the most potent candidate in scavenging DPPH free radicals. From the result obtained, *P. amaryllifolius* extracts showed increasing activity with increasing polarity of the solvent. This could be linked with the TPC results because based on Pearson correlation test conducted, there is a significant correlation between TPC and DPPH assay. Therefore, this suggests that phenolic compounds may contribute to DPPH radical scavenging effects in *P. amaryllifolius* extracts. Thus, this showed that extraction using different solvents lead to different capability of antioxidant scavenging activity due to varying phytochemical composition present in each extract. Few other studies also proved a significant correlation between TPC and DPPH assay (Ng *et al.*, 2012; Tiveron *et al.*, 2012; Ablat *et al.*, 2014; Saha & Verma, 2016).

Ferric reducing antioxidant power (FRAP) assay is measured via the principle of reducing ferric (III) to ferrous (II) ion (Sahgal *et al.,* 2009). FRAP assay of *P. amaryllifolius* extracts. also exhibited increasing activity as the solvent polarity increased. Just like DPPH, this result associated with TPC as FRAP assay showed significant correlation with TPC. This result is in agreement with other studies that discovered positive significant correlation between TPC and FRAP assay (Gan *et al.,* 2013; Ku *et al.,* 2014; Hanis Mastura *et al.,* 2017). Study by Kabouche *et al.* (2007) stated that a few terpenoids displayed effective reducing power in FRAP aasay. As terpenoids were detected in all five extracts during TLC experiment, this advocates the role of terpenoids in *P. amaryllifolius* as reductants that donates an electron, transforming Fe^{3+} to Fe^{2+}.

Metal chelating activity evaluates the ability of antioxidant in chelating ferrous ion. The system is dose dependent whereby higher sample concentration results in higher chelating activity. In our body, ferrous ion (Fe^{2+}) leads to the formation of reactive oxygen species which can harm body cells (Yamaguchi *et al.,* 1988). The metal chelating mechanism involves the formation of ferrous-ferrozine complex from the chelating activity of Fe^{2+} by extracts. In this assay *P. amaryllifolius* extracts were studied for its ability to compete with ferrozine for ferrous ion and the chelating ability was measured via absorbance detected at 562 nm with the formation of Fe^{2+}- ferrozine complex. Andjelković *et al.* (2006) stated that phenolics in plants may not be able to chelate iron as good as EDTA. The order of ferrous ion chelating activity is: PA-W > PA-H > PA-M > PA-PE > PA-C. The result from this assay showed no correlation with TPC. This implies that the amount of phenolics in *P. amaryllifolius* extracts did not affect metal chelating activity. Study by Taherkhani (2016) also found that TPC and metal chelation activity possessed no correlation. On the other hand, metal chelating

activity showed negative correlation with TFC. This suggests that flavonoids do not appear to be effective ferrous ion chelators.

Hydrogen peroxide is non reactive, however it may possess toxic effect to cells by transforming into hydroxyl radicals that reacts with biomolecules. This results in the destruction of body tissues as well as the death of cells (Khan *et al.,* 2012). Result from this assay proposed that there is a significant correlation between TPC and hydrogen peroxide scavenging activity, similar to DPPH assay. This indicates that high phenolic content lead to high scavenging activity of hydroxyl radicals. A number of studies also revealed the presence of strong correlation between TPC and hydrogen peroxide scavenging activity (Khan *et al.,* 2012; Chakraborty *et al.,* 2013; Kumar *et al.,* 2017). Meanwhile, TFC exhibited negative correlation with H_2O_2 scavenging activity. Tiveron *et al.* (2012) stated that each penolic and non-phenolic compounds possessed varying antioxidant potential by which high amount of particular compounds will not necessarily assure potent antioxidant effects.

5.5 *In vitro* xanthine oxidase inhibitory activity

In vitro study was conducted to determine the ability of *P. amaryllifolius* extracts to retard the activity of xanthine oxidase enzyme. According to Umamaheswari *et al.* (2009) phenols and flavonoids present in extracts have the ability to express inhibitory activity of xanthine oxidase. The evaluation was conducted at concentration range of 6.25–100 μg/mL. The highest xanthine oxidase inhibitory activity was shown by PA-W, followed by PA-M and lastly PA-H. Two other extracts which are PA-PE and PA-C have shown no inhibition activity. This is possibly because of inadequate specific compounds that possess xanthine oxidase inhibition capability in the extracts (Azmi *et al.,* 2012).

5.6 *In vivo* xanthine oxidase inhibitory activity

PA-W was selected and screened for *in vivo* hypouricemic activity. Azmi *et al.* (2012) stated that in a large scale, high amount of solvents are needed and it is wiser to use the most economical solvent in the extraction process while considering its safety measure. Since methanol is toxic to the body as it can interfere the physiological acid-base balance, solvents with lower toxicity with same potential should be utilized wherever possible. Water as the world's universal solvent is crucial for every living thing. It is easily obtainable, pretty well significant, and cost effective.

In hypouricemic study, the ability of PA-W to impede hyperuricemia induced by oxonic acid potassium salt was studied using Spraque Dawley rats. Based on the results obtained, PA-W exhibited significant hypouricemic activity for both high (1000 mg/kg) and low (500 mg/kg) dosage. Based on the results, allopurinol still possessed the most outstanding effect on both *in vitro* xanthine oxidase inhibitory activity and *in vivo* hypouricemic activity. Allopurinol is widely used and easily available to treat hyperuricemia, gout and other inflammatory ailments. However, due to hypersensitivity, intolerance, and failure of the treatment on certain patients (Lü *et al.*, 2013) alternative therapeutic agents especially xanthine inhibitors in natural compounds are recommended as it displays fewer side effects (Wong *et al.*, 2014). In this experiment, dose-dependent PA-W possessed practically comparable effect with allopurinol in reducing serum uric acid level and XO activity. Umamaheswari *et al.* (2007) stated that the presence of phytoconstituents in extracts along with their anti-inflammatory and antioxidant activities manifest a notable XO inhibitory activity and hypouricemic activity. However, TPC showed no correlation with *in vitro* xanthine oxidase inhibitory activity in this study. According to Kostić *et al.* (2015) the way to treat gout and hyperuricemia is by elevating the uric acid excretion or minimizing the production of uric acid. Since PA-W has significantly and successfully reduced the uric acid level and

inhibited XO activity in hyperuricemic rats serum, this indicates that the use of PA-W may be beneficial in gout and hyperuricemia therapy. This contributes to the beginning of further examination on this species to isolate its active constituents and invention of drug.

CHAPTER 6: CONCLUSION

Pandanus amaryllifolius Roxb. extracts extracted with hexane, petroleum ether, chloroform, methanol and distilled water contain a number of phytochemicals which include phenolic and flavonoid compounds. These bioactive compounds possess antioxidant activities and display an outstanding impact in healing numerous diseases. In antioxidant assays, extracts that were extracted using different solvent polarity displayed different level of antioxidant capability. PA-W acts as the most potent candidate in this research study. It exhibited the highest effectiveness in both antioxidant activity and xanthine oxidase inhibitory activity compared to other extracts. PA-W and PA-PE contributed the highest TPC and TFC, respectively. PA-W possessed the highest inhibition in DPPH radical scavenging activity, metal chelating activity, and hydrogen peroxide scavenging activity. PA-M showed the most potent reducing power in FRAP assay. *In vitro* xanthine oxidase inhibitory activity displayed PA-W as the strongest xanthine oxidase inhibitor. PA-W also had significantly reduced serum uric acid level and inhibited xanthine oxidase activity in *in vivo* xanthine oxidase inhibitory. The results obtained from this study suggest *P. amaryllifolius* as a novel xanthine oxidase inhibitor derived from natural sources that shows potent activity and low toxicity in the future.

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