STUDIES ON XANTHINE OXIDASE INHIBITORY ACTIVITY OF
Plumeria rubra Linn FLOWER

SITI SARWANI PUTRI BINTI MOHAMED ISA

DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENT FOR THE PARTIAL DEGREE OF MASTER OF BIOTECHNOLOGY

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ABSTRACT

Plumeria rubra Linn is locally known in Malaysia as "kemboja" and belongs to family Apocynaceae. It has been used traditionally in Southeast Asia for the treatment of malaria, diabetes, analgesic to relieve fever and alleviate arthritis. The Thin Layer Chromatography (TLC) profile of PR−HE, PR−CE, PR−ME and PR−WE extracts of showed the presence of essential oils, phenols, flavonoids and terpenoids. The analysis red flowers extracts with LCMS/MS displayed that it contains 3− caffeyolquinic acid, 5− caffeoquinic acid, 1,3− dicaffeoquinic acid, chlorogenic acid, citric acid, 3,3− di− O− methylellagic acid, kaempferol−3−O−glucoside, kaempferol−3−rutinoside, kaempferol, quercetin 3−O− α−L−arabinopyranoside, quercetin, quinic acid and rutin. The TPC and TFC contents were highest in PR-ME extract at 184.632 mg GAE/g and 203.2.2 mg QE/g, respectively. In the six different antioxidant activity assays, PR−ME extract exhibited the best DPPH scavenging activity at IC_{50} value at 59.385 µg/ml, highest reducing power toward ferric ions which was 4.950 mmol Fe^{2+}/g extract, highest metal chelating activity at IC_{50} value at 185.559 µg/mL, highest in hydrogen peroxide scavenging activity at IC_{50} value at 248.376µg/mL, highest in nitric oxide scavenging activity at IC_{50} value at 62.096 µg/mL and also highest in superoxide radical scavenging activity at IC_{50} value at 54.773 µg/mL. The result of XO inhibitory activity *in vitro* assay revealed that the PR−ME extract possesses highest inhibition effects with the IC_{50} value was 23.91 µg/mL. The acute toxicity test among the males and females rats demonstrated the PR−ME extract were safe up to a maximum dose of 4000 mg/kg body weight due to no mortality and sign of toxicity occurred within two weeks of experiment. Administration of PR−ME extract at doses of 400 and 200 mg/kg to the rats significantly reduced serum uric acid level by 67.5 % and 28.9 %, respectively when compared to gout control group. Furthermore, the significant

inhibitory actions on the XO activity in serum were showed at doses at 400 and 200 mg/kg in which were 7.18 mU/mL by 43.77 % and 8.73 mU/mL by 31.64 %, respectively. The result of in vivo study indicated that serum XO activity was correlated with liver XO activity. XO activity in liver was significantly inhibited by PR−ME extract at doses of 400 and 200 mg/kg, which were 7.76 mU/mL by 31.64 % and 9.88 mU/ml by 16.06 %, respectively. However, PR−ME extract did not show any XO inhibition effects in normal rats. Moreover, no significant difference of body weight between treated rats and non-treated rats within 7 days of extract administration. In conclusion, PR−ME extract from *P. rubra* red flower contained active phytochemical compounds as detected in LCMS/MS has contributed to inhibition of XO activity in *in vitro* and *in vivo* which also had partly mediated to the urate lowering effects in serum as well as the potential to scavenged free radical intermediates and superoxide byproducts.

ABSTRAK

Plumeria rubra Linn dikenali di Malaysia sebagai "kemboja" dan terdiri dari keluarga Apocynacea. Ia digunakan secara tradisional di Asia Tenggara dalam rawatan malaria, kencing manis, sebagai analgesik untuk meredakan demam dan arthritis. Kromatografi lapisan nipis (TLC) dari ekstrak PR–HE, PR–CE, PR–ME dan PR–WE menunjukkan kehadiran minyak pati, phenols, flavonoid dan terpenoids. Analisis ekstrak bunga merah dari *P. rubra* melalui LCMS/MS mempamerkan kehadiran 3–O–caffeyolquinic acid, 5–O–caffeoquinic acid, 1,3–dicaffeoquinic acid, chlorogenic acid, citric acid, 3,3–di–O–methylellagic acid, kaempferol–3–O–glucoside, kaempferol–3–rutinoside, kaempferol, quercetin 3–O–α–L–arabinopyranoside, quercetin, quinic acid dan rutin. Jumlah kandungan TPC dan TFC yang tertinggi dikesan dalam PR–ME iaitu masing-masing sebanyak 184.632 mg GAE/g dan 203.2.2 1 mg QE/g. Dalam enam cerakin aktiviti antioksidan yang berbeza, PR–ME menunjukkan aktiviti pemerangkapan DPPH tertinggi dengan nilai IC_{50} sebanyak 59.385 µg/mL, nilai tertinggi dalam keupayaan mengurangkan kuasa ke arah ion ferric iaitu 4.950 extract/g mmol Fe^{2+}, aktiviti pengkelatan logam yang tertinggi dengan nilai IC_{50} sebanyak 185.559 µg/mL, nilai tertinggi dalam pemerangkapan radikal hidrogen peroksida dengan nilai IC_{50} sebanyak 248.376 µg/mL, nilai tertinggi dalam pemerangkapan radikal nitrik oksida dengan nilai IC_{50} sebanyak 62.096 µg/mL dan juga nilai tertinggi dalam pemerangkapan radikal superoksida dengan nilai IC_{50} sebanyak 54.773 µg/mL. Aktivity perencatan enzim XO secara *in vitro* mendedahkan PR–ME mempunyai kesan perencatan yang tertinggi dengan nilai IC_{50} sebanyak 23.91 µg/mL. Ujian ketoksikan akut menunjukkan PR–ME didapati selamat diberikan kepada haiwan sehingga dos maksimum 4000 mg/kg berat tikus, berdasarkan tiada kematian dan ciri- ciri ketoksikan yang berlaku pada tikus sepanjang dua minggu eksperimen. Kedua- dua dos PR–ME iaitu 400 dan 200 mg/kg telah

menurunkan kadar asid urik dalam serum tikus secara ketara iaitu masing-masing sebanyak 67.5 % dan 28.9 %, jika dibandingkan dengan kumpulan tikus dalam kawalan gout. Tambahan pula, berlaku perencatan secara ketara terhadap aktiviti enzim XO di dalam serum melalui pemberian dos 400 dan 200 mg/kg PR–ME, di mana masing-masing memberi nilai aktiviti sebanyak 7.182 mU/mL iaitu peratus perencatan adalah 43.77 % dan 8.731 mU/ml iaitu peratus perencatan adalah 31.64 %. Kajian aktiviti XO secara *in vivo* mendapati aktiviti enzim XO dalam serum mempunyai korelasi dengan activiti enzim XO dalam hati. Aktiviti perencatan enzim XO dalam hati berlaku secara ketara oleh dos 400 mg/kg iaitu sebanyak 7.76 mU/mL dan peratus perencatan ialah 34.07 % manakala dos 200 mg/kg memberi nilai aktiviti sebanyak 9.88 mU/mL dan peratus perencatan ialah 16.06 %. Namun begitu, tiada kesan perencatan XO ditunjukan oleh PR–ME kepada kumpulan tikus kawalan normal. Malahan, tiada perubahan yang ketara didapati pada berat badan tikus dalam kumpulan rawatan dan dalam kumpulan kawalan sepanjang 7 hari eksperimen. Kesimpulannya, PR–ME dari ekstrak bunga merah *P.rubra* mengandungi sebatian fitokimia aktif yang dikesan melalui analisis LCMS/MS didapati telah menyumbang terhadap aktiviti perencatan enzim XO secara *in vitro* dan *in vivo*, menjadi pengantara kepada sebahagian mekanisme penurunan urate di dalam serum dan berupaya menyingkirkan pengantara radikal bebas dan produk superoksida.

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LIST OF SYMBOLS AND ABBREVIATIONS

ABCG2	Human ATP-binding cassete; Secretory urate transporter
Abs	Absorbance
Ala	Alanine
ALCL$_3$	Aluminium Chloride
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
Arg	Arginine
ATP	Adenosine triphosphate
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
CCL2	C-C motif chemokine ligand 2
CMC	Carboxymethylcellulose
DMSO	Dimethyl sulfoxide
DNA	Deoxy ribonucleic acid
DPPH	2,2-diphenyl-1- picrylhydrazyl
EDTA	Ethylenediamine tetraacetic acid
EPR	Electron paramagnetic resonance
FAD	Flavine adenine dinucleotide
FC	Folin ciocalteu reagent
FeCl$_2$	Ferum chloride
FeCl$_3$	Ferric chloride
FeSO$_4$	Ferum sulphate
FeS	Iron sulphur center
FRAP	Ferric Reducing Anti−Antioxidant Power
FZ	Ferrozine
Glu	Glutamic acid
GMP	Guanosine monophosphate
GSH	Gluthathione
GSSG	Oxidized gluthathione
GSSR	Gluthathione reductase
H$_2$O$_2$	Hydrogen peroxide
HCl	Hydrochloric acid

HGPRT	Hypoxanthine-Guanine phosphoribosyltransferase
IC_{50}	Half maximal inhibitory
IgM	Immunoglobulin M
IL	Interleukin
IMP	Inosine monophosphate
iNOS	Inducible nitric oxide synthetase
K^+	Potassium ions
KH_2PO_4	Potassium phosphate (monobasic)
K_2HPO_4	Potassium phosphate (dibasic)
LD_{50}	Median lethal concentration
LCMS/MS	Liquid Chromatography Mass Spectrometry/ Mass Spectrometry
LRR	Leucine Rich repeat
Mo-Co	Molybdopterin cofactor
MSU	Monosodium urate crystals
MRP4	Multidrug resistance-associated protein 4
NaCl	Sodium Chloride
Na_2CO_3	Sodium Carbonate
NAD	Nicotinamide adenine dinucleotide
NALP3	NACHT, LRR and PYD domains-containing protein 3
$NaNO_2$	Sodium nitrate
NaOH	Sodium hydroxide
NBT	Nitro Blue tetrazolium
NHANES III	Third National Health and Nutrition Examination Survey
NO	Nitric oxide radical
NSAID	Non-steroidal anti-inflammatory drugs
$O_2{}^{\cdot-}$	Superoxide radical
$ONOO^-$	Peroxide radical
OAT	Organic ion transport
OH^-	Hydroxyl radical
P2X7	P2X-purine receptor 7
Phe	Phenylalanine
PMS	Phenazine methylsulphate- nicotinamide adenine
PO	Potassium oxonate
PR-CE	Chloroform extract of *P.rubra* flower
PR-HE	Hexane extract of *P.rubra* flower

PR-ME	Methanol extract of *P.rubra* flower
PR-WE	Water extract of *P.rubra* flower
PRPP	Phosphoribosyl pyrophosphate
PYD	Pyrin domain
Rf	Distance a compound moves in chromatography relative to the solvent front
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SD	Sprawgue Dawley rats
SLC 2A9 (GLU T9)	Fructose transporter
$Na_2[Fe(CN)5NO] \cdot 2H_2O$	Sodium nitroferricyanide
TENS	Toxic Epidermal Necrolysis syndrome
TFC	Total Flavonoid content
Thr	Thrionine
TLC	Thin layer chromatography
TLR	Toll-Like receptor
TNF	Tumour Necrosis factor
TPC	Total Phenolic content
TPTZ	Tripyridyltriazine ;2,4,6-Tri(2-pyridyl)-s-triazine,
UAT	Urate transporter
ULT	Urate lowering therapy
URATI 1	Urate transporter 1
UV	Ultraviolet
XO	Xanthine oxidase
XDH	xanthine dehydrogenase
XOR	xanthine oxidase reductase
° C	Celcius degree
v/v	Volume over volume
µL	Microliter
nm	Nanometer
mL	Mililiter
g	Gram
kg	Kilogram
mU	Miliunit
pKa	Measure of acid strength

α	Alfa
mg	Milligram
dL	Decilitre
Mmol	Milimolar
µg	Microgram

CHAPTER 1

INTRODUCTION

Gout is one of diseases that has been investigated for decades. According to epidemiological studies from different countries revealed that rising of the prevalence and incidence of gout has been resulting to a substantial economic burden (Roddy *et al.* 2007; Wu *et al.* 2008). It is most prevalent form of inflammatory arthritis and is associated with impaired quality of life. The development of gout is derived from the key predictor, hyperuricemia, which is elevated uric acid above super−saturation concentrations (6.8 mg/dl at physiological pH and temperature) leading to formation monosodium urate (MSU) crystals within joints and subcutaneous tissues associated to development of painful attacks of gouty arthritis (Dalbeth *et al.* 2008).

Xanthine oxidase (XO) which is present in significant concentration in liver and intestines, has been considered as major contributor to gout disease. The formation of uric acid occur from the final biosynthesis in purine catabolic pathway which it responsible to catalyzes the oxidation process of hypoxanthine to xanthine and further, xanthine to uric acid. The overproduction and/or underexcretion of this uric acid lead to the major contributor of incidence of gout which is hyperuricemia (Rasaratnam & Christophidis, 1995). Moreover, during the reoxidation of this enzyme, molecular oxygen acts as electron acceptor leads to reactive oxygen species production including superoxide radical and hydrogen peroxide. Consequently, elevated oxidative stress has been reported in gouty patient (Urano *et al.* 2002). Therefore, (XO) is gaining interest among researchers in worldwide due to its important biological source of oxygen−derived free radicals which contribute to oxidative damage to living tissues involved in many pathological processes including the inflammation, atherosclerosis, cancer, aging and

gout (Chiang *et al.* 1994; Cos *et al.* 1998; Pacher *et al.* 2006). *In−vitro* bioassays is pre-requisite in order to basis screening sample for XO inhibition, as inhibitors of XO may be potentially useful for new medicament of gout or other XO induced diseases (Goodman Gilman *et al.* 2006). Therefore, the capability of bioactive compounds to inhibit the activity of XO might be able to decreased uric acid level and also in ROS production, so that this phytochemical compound may alleviate inflammation, then; it serves as anti-hyperuricemic and antioxidative effects.

The ultimate formation of uric acid in man depends on the activity of enzyme, XO, on hypoxanthine and xanthine. This concerns the common treatment of gout is allopurinol or (4 − hydroxypyrazolo [3, 4 − d] pyrimidine) (Hak & Choi, 2008). Allopurinol is the kind of XO inhibitors class of drugs, which is most commonly approach in order to lower urate levels in serum and urine. In addition, allopurinol is effective in decreasing flares and tophi, particularly among patients in whom target urate levels are achieved. Allopurinol is slowly oxidized to its active metabolite; oxypurinol as a xanthine analogues, act as more potent XO inhibitor in order to impairs the conversion of hypoxanthine to xanthine and then conversion of xanthine to uric acid. It showed beneficial effects in the treatment of hyperuricemia both in experimental animal models and in small-scale human clinical trials. Although allopurinol has been successfully used as urate- lowering therapy, but it is not the appropriate choice in treating acute gouty athritis (Dubchak & Falasca, 2010). Instead, allopurinol generates superoxide (Berry & Hare, 2004) and some people develop rash as they are allergic to allopurinol (Kong *et al.* 2000). Severe reactions also occur including liver function abnormalities (Fields *et al.* 1996), a fatal complication known as "allopurinol hypersensitivity syndrome" (Kelso & Keating, 1996; Kong *et al.* 2000) and adverse drug reactions such as Toxic Epidermal Necrolysis syndrome (TENS) (Pacher *et al.* 2006). Furthermore, safety of using allopurinol in children and during pregnancy has not been established (Borges *et al.*

2002). Non- steroidal anti–inflammatory drug (NSAID) such as indomethacin are often used as first–line therapy to treat acute inflammation in gout.

In addition, this drug also contributed to adverse effects including gastrointestinal toxicity, renal toxicity or gastrointestinal bleeding (Sabina *et al.* 2010) .Therefore, many research to develop an alternative medicine which for hyperuricemia and gout has focused on the screening of natural substances due to the adverse side effects of allopurinol, which so far, remains the only prescription for the disease. Understanding these facts, has motivated the recent studies which focus on biological activities such as new anti–inflammatory and hypouricemic effect, including xanthine oxidase inhibitors, from natural sources which could be useful in gout treatment (Zhu *et al.* 2004; Mo *et al.* 2007; Ahmad *et al.* 2008; Liu *et al.* 2008; Haidari *et al.* 2008, 2009; Sabina *et al.* 2010; Huang *et al.* 2011).

Use of plants for health improvement was practiced in Chinese medicine since 4000–6000 years ago (Farkas *et al.* 1986). Flavonoids belong to a group of natural substances with variable phenolic structures and are found in fruit, vegetables, grains, bark, roots, stems and flowers. Their contribution to physiological functions such as seed maturation and dormancy has already been established. Flavonoids have probably existed in the plant kingdom for over one billion years. Therefore, this long interaction between flavonoids compound and humans with its benefits has encouraged much interest of many researchers over the world to study the biochemical and physiological activities of these phytochemical. Researches on flavonoids have suggested these compounds might act as active inhibitors for XO (Cos *et al.* 1998). Flavonoids also are widely distributed polyphenolic compounds which acts as free radical scavengers by fast donation of hydrogen atoms to free radicals. Plant has been used as experimental material in this study, since medicinal plants have always been significant for pharmacological research and drug development. The flavonoids contents in *Plumeria rubra* red flowers would

become the new alternatives to allopurinol with increased therapeutic activity and fewer side effects. The aim of this study is, therefore, to investigate the potential of phytochemical bioactive compounds from *P.rubra* flower as XO inhibitor for gout treatment *in vitro* assay as well as *in vivo* model using experimental gout model.

The selected ornamental plant, *P.rubra* is currently gaining interest by the scientific researchers due to its therapeutic value. In the present study, the Indian medicinal plant, *P.rubra* flowers was selected based on ethnomedicinal use in gout and arthritis (Burkill, 1966), inflammation, constipation (Hamburger *et al.* 1991; Gupta *et al.* 2006) and hypolipidemic (Merina *et al.* 2010). Despite the widely popular use of *P.rubra* flowers, the available scientific information about the potential effects of *P.rubra* flowers in animal models of gout study is limited. Although there are many studies demonstrated XO inhibitory effects from various medicinal plants and also phytochemical bioactive compounds, there is no previous research has been reported on XO inhibitory activity from *P.rubra*. Thus, the inhibitory effects of bioactive compounds from the red flower of *P.rubra* on XO activity are worth to be evaluated. Therefore, the present study was conducted to identify and evaluate the phytochemical bioactive compounds, in red flowers of *P.rubra* as a XO inhibitors and as a potential of alternative drugs of plant origin for gout treatment.

1.1 General Objective

The general objective of this study is to investigate the potential of bioactive compounds in *P.rubra* red flower in inhibition of XO activity in *in vitro* and *in vivo* model.

1.2 Specific Objectives

i. To detect and identify the phytochemical bioactive compounds in extracts using Thin Layer Chromatography (TLC) and LCMS.

ii. To evaluate the antioxidant activity of extracts.

iii. To evaluate xanthine oxidase (XO) inhibitory activity of extracts in *in vitro* model.

iv. To evaluate xanthine oxidase (XO) inhibitory activity of extracts in *in vivo* model.

CHAPTER 2

LITERATURE REVIEW

2.1 Gout: Global scenario and Background

Gout is known as inflammatory disorder caused by the deposition of monosodium urate (MSU) crystals in articular and periarticular tissues (Choi, 2005). In addition, gout is a common biochemical abnormality with an estimated prevalence of 8.4 cases per 1,000 persons (Hwang & Saag, 2013) and is associated with painful arthritis attacks and the development of metabolic syndromes (Choi *et al.* 2007) and nephropathy (Avram and Krishnan, 2008). These crystals cause an acute inflammatory response and can induce a permanent tissue damage which is characterized by the appearance of ulceration of the joint cartilage, marginal osteophytosis, geodic and erosive lesions and chronic inflammation of synovial membrane (Dalbeth, 2005; Corrado *et al.* 2006).

Gout is already became a common medical problem which occurred in up to 1 % of individuals in Western countries. Estimations from the Third National Health and Nutrition Examination Survey (NHANES III) indicate that 0.5 % of the total population from US has suffered from a gout attack. Gout is currently considered to be the most common form of inflammatory arthritis in men over 40 years old exceeding rheumatoid arthritis (Weaver, 2008). Moreover, the epidemiological studies showed the prevalence of gout is increasing not only in elderly but also is higher among men than among women, with a ratio of 4 to 1 overall. However, this sex disparity decreases at older ages, at least in part because of declining levels of estrogen, which has uricosuric effects in women (Wallace *et al.* 2004: Miao *et al.* 2008: Doherty, 2009: Zhu *et al.* 2011). Evidently, it represents the most frequent chronic inflammatory arthritis in men and an

increasing cause of arthritis in women (Roddy & Doherty, 2010: Zhu *et al.* 2011). The rising of incidence gout is closely associated with unhealthy life-style choices, including of increased intake of high purine diets which particularly meats and seafoods, alcohol which particularly beer and spirits, and fructose–sweetened drinks that caused metabolic syndrome with obesity, longevity, insulin resistance and hyperlipidemia (Choi *et al.* 2004; Choi *et al.* 2007; Choi *et al.* 2008: Wu *et al.* 2008; Neogi, 2011; Singh *et al.* 2011) and also the number of age–related risk factors including an increase in the prevalence of renal failure and diuretic–treated hypertension (Choi *et al.* 2004). Gout is strongly associated with obesity and metabolic syndrome. Adiposity and weight gain are important risk factors for development of gout, while weight loss is protective (Choi, 2005). Besides this, recipients of organ transplants also have an increased risk of gout due to use of cyclosporine and other antirejection drugs (Baroletti *et al.* 2004).

2.2 Uric acid

Uric acid is the final product of purine metabolism and these catabolic steps are catalyzed mainly by XO enzyme in human. Instead, in most mammals, uric acid is degraded by uricase to allantoin which is highly soluble and carbon dioxide, then readily excreted in the urine with no obvious adverse effects. Uricase is inactive in human due to evolution which including diverse mutations, probably environmentally driven gave place to a non-functional gene. During the Miocene period, two parallel mutations occurred in early hominids and humans that disabled uricase gene, resulting in higher serum uric acid concentrations. The parallel mutation suggests the inactivating uricase gene was confer some advantageous to early hominids and humans, possibly due to one, or a combination of the antioxidant activity of uric acid compensating for vitamin C

deficiency; the ability of uric acid to maintain blood pressure under low- salt dietary condition; the adjuvant activity of uric acid (Wu *et al.*1992; Dalbeth *et al.* 2008).

2.2.1 Chemistry of Uric acid

Uric acid or IUPAC name as 2, 6, 8−trioxypurine (Figure 2.1) is a weak acid with 2 ionization constants (pK_{a1}= 5.75 and pK_{a2}=10.3). The solubility product of monosodium urate increases with increasing ionic strength. At pH 7.7 such as in serum or synovial fluids, about 98 % of uric acid is ionized as monosodium urate whereas at lower pH such in the urine, it exists mostly in free form. The chemical properties of uric acid is significant to the development of gout disease which involved its capability to form crystals and its relative insolubility in non-alkaline solutions (Seegmiller *et al.* 1963).
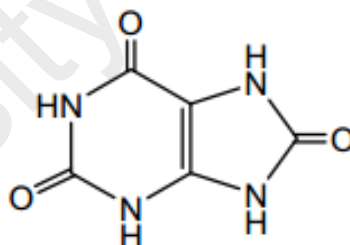
Figure 2.1. Chemical structure of uric acid

Adapted from Seegmiller *et al.* 1963

2.2.2 Formation of Uric acid

Uric acid, produced mostly in the liver, and the end product of exogenous and endogenous purine metabolism. The exogenous sources for uric acid are the purine and purine precursors from the diets. A diet rich in animal proteins contributes significantly to the purine pool and subsequent uric acid formation by a series of enzymatic reactions involving xanthine oxidase as the final step. The endogenous production of uric acid from de novo purine synthesis, and tissue catabolism breakdown of nucleic acids and nucleotides (salvage synthesis) under normal circumstances (Newcombe, 1975).

In normal circumstances salvage predominates over synthesis, but synthesis is particularly active in tissues with a high rate of cell turnover such as in gut epithelium, skin, bone marrow and etc (Stone & Simmonds, 1991). Ribose-5-phosphate, derived from glycidic metabolism, is converted to phosphoribosyl pyrophosphate (PRPP) via the PRPP synthetase and then to inosine monophosphate (Cammalleri & Malaguarnera, 2007). This intermediate compound yields adenosine monophosphate (AMP) and guanosine monophosphate, the purinic nucleotides used in DNA and RNA synthesis, as well as inosine. The latter is converted by the purine nucleoside phosphorylase to hypoxanthine. Xanthine oxidase (XO), an enzyme inhibited by allopurinol, converts hypoxanthine to xanthine and subsequently xanthine to uric acid (Figure 2.2). Hypoxanthine and guanine may enter a salvage pathway through the activity of hypoxanthine – guanine phosphoribosyltransferase (HGPRT), an enzyme that reconverts these purine bases into their respective nucleotides. Key enzymes that cause abnormal uric acid levels include: PRPP synthetase, purine nucleoside phosphorylase, XO and HGPRT (Wilcox, 1996).

Ribose-5-Phosphate

PRPP
Synthetase

PRPP

ATP

AMP ← IMP → GMP

APRT +Adenosine   Inosine   HGPRT+   Guanosine HGPRT
PRPP   PRPP   +PRPP
        Purine Nucleoside
        Phosphorylase

—Adenine   Hypoxanthine —   Guanine—

                        XO

                  Xanthine ←

                        XO —| *Allopurinol*

                  Uric Acid

                        | *Uricase*

                  Allantoin
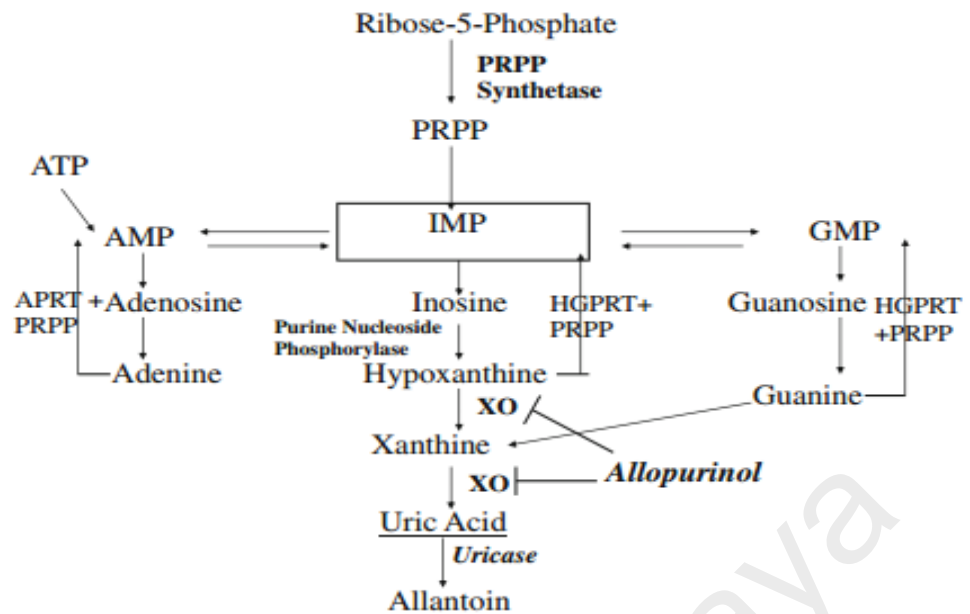
Figure 2.2. Pathway of purine metabolism. A schematic diagram of purine metabolism including the enzymes involved in uric acid synthesis and degradation. Key enzymes include PRPP synthetase, purine nucleoside phosphorylase, XO and HGPRT. PRPP Phosphoribosyl pyrophosphate, ATP Adenosine triphosphate, AMP Adenosine monophosphate, IMP Inosine monophosphate, GMP Guanosine monophosphate, APRT Adenine phosphoribosyltransferase, HGPRT Hypoxanthine-guanine phosphoribosyltransferase, XO Xanthine oxidase Adapted from Fathallah-Shaykh & Cramer, (2014).

2.2.3 Excretion of Uric acid

The homeostasis of blood urate levels is maintained by the balance between purine biosynthesis pathway and excretion. Primary regulation of uric acid excretion is by renal-handling which acts as uric acid clearance and accounting for two-thirds of its elimination. The remaining one-third of uric acid excretion are occurs in intestine skin, hair and nails account. In the intestine bacteria catabolize uric acid into carbon dioxide and ammonia, which are then eliminated as intestinal air or absorbed and excreted in the urine.

Renal handling of uric acid is complex whereby 95 % of plasma uric acid is filtered through glomerulus as uric acid. In humans, net reabsorption of uric acid into the blood predominates owing to less excretion of uric acid than is filtered at the

glomerulus. Passive back diffusion of secreted uric acid occurs with the result only with only 10 % of that filtered is finally excreted in the urine. This renal exchange is mediated by specialized molecule expressed in renal proximal tubule cells (Dalbeth & Merriman, 2008) (Figure 2.3). The identified molecule are included the fructose transporter SLC2A9 (GLUT9), urate ransporter 1 (URAT1), organic anion transporter 1, 3, 4 (OAT1, OAT3, OAT4), multi-drug resistant protein 4 (MRP4), sodium coupled monocarboxyl transporters SMCT1, 2 and human ATP – binding cassete, subfamily G, (ABCG2) (Enomoto *et al.* 2002; Caulfield *et al.* 2008; Woodward *et al.* 2009). URAT1 and SLC2A9 (GLUT9) are members of the organic acid transporter (OAT) family and have predominant effects on serum urate level. SLC2A9 gene is glucose transporter 9 (GLUT9) which transports the sugars glucose and fructose from the proximal tubule lumen across the apical and basolateral membranes, which may be pertinent to the dietary influences of these compounds on hyperuricemia and gout. SLC2A9 is inhibited by the uricosuric agent, benzbromarone but not by the commonly used uricosuric agent probenecid. URAT1 is highly specific for urate and mediates solute concentration-dependent bidirectional exchange of urate for a variety of endogenous and drug anions known to affect renal uric acid transport. URAT1 also inhibited by the uricosuric agent, benzbromarone and probenecid. OAT4 is a low-affinity assymetric urate transporter that facilitates diuretic associated hyperuricemia, also inhibited by benzbromarone and probenecid. MRP4 is control ATP–dependent urate extrusion from the cells into the tubule lumen and thus contribute to urate excretion. ABCG2 is a secretory urate transporter in the proximal tubule, which the activity of uricosuric agent on ABCG2 still not known. In other words, these uric acid transportasome have been associated with hyperuricemia and gout (Enomoto *et al.* 2002; Caulfield *et al.* 2008; Woodward *et al.* 2009; Vitart *et al.* 2008). Taniguchi *et al.* (2005) reported that the main cause of the renal impairment of urate excretion is due to the genetic defects.
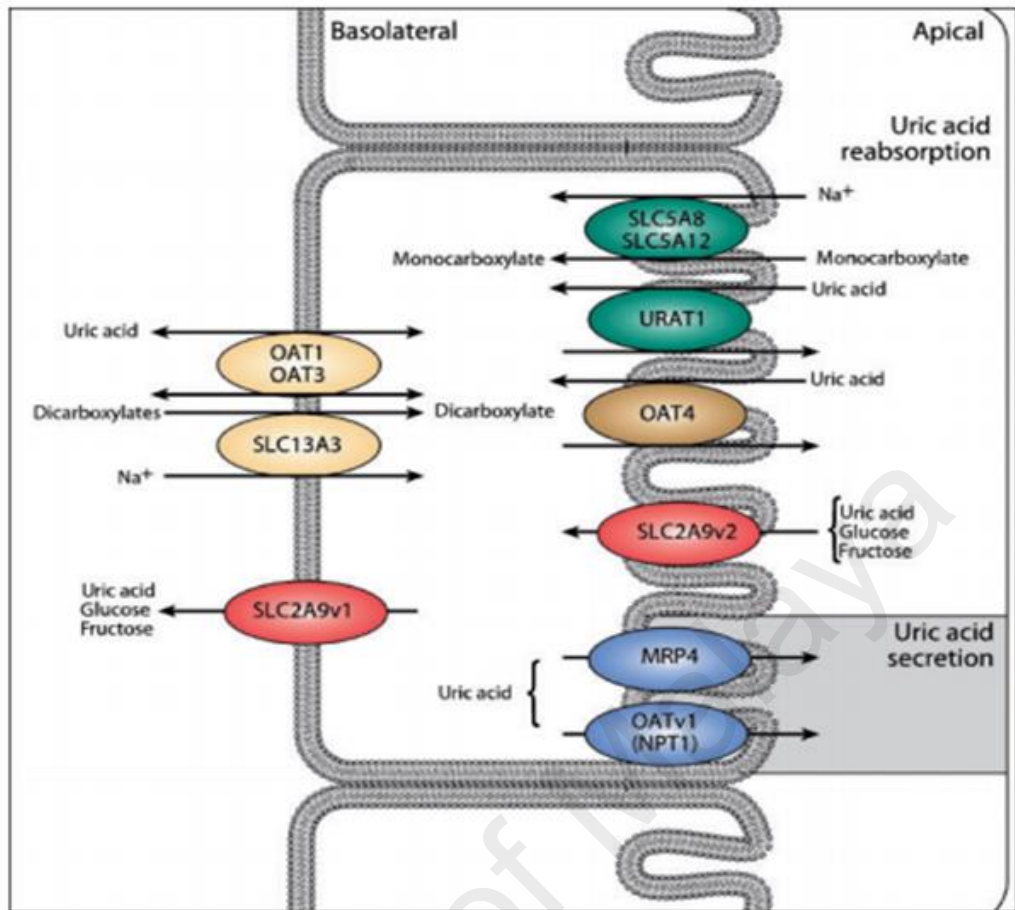
Figure 2.3. The uric acid transportasome. Monocarboxylates accumulate in the tubular cell through sodium-dependent monocarboxylate transporters SLC5A8 and SLC5A12, and dicarboxylates through SLC13A3. Uric acid enters the cell in exchange for monocarboxylatevia apical URAT1 and for dicarboxylate via apical OAT4. Apical SLC2A9v2 plays a significant role in uric acid reabsorption, the reabsorbed uric acid exiting the cell through basolateral SLC2A9v1 Adapted from Merriman & Dalbeth, (2011).

2.3 Gout and Hyperuricemia

Hyperuricemia is prerequisite of gout and identified when serum uric acid/urate (sUA) levels are above 7 mg/dl (0.42 mmol/l) in men and 6.5 mg/dl (0.387 mmol/l) in women, approximately the saturation point for urate solubility at physiologic temperature and pH. Gout may appeared in varying duration depends on the degree of hyperuricemia and other factors (Mikuls *et al.* 2005) Many studies had evident the close relationship of serum uric acid with an increased risk of gout episodes (Cleland *et al.* 1995; Shoji *et al.* 2004). Asymptomatic hyperuricemia is considered in patients with hyperuricemia but without clinical signs of gout or lithiasis (Vazquez-Mellado *et al.* 2004; Choi, 2005). Ruilope and Garcia-Puig (2001) stated that blood uric acid level of more than 9 mg/dL considered as a severely hyperuricemic condition.

Hyperuricemia results from overproduction or underexcretion of uric acid. About 80 to 90 % of the patients with hyperuricemia or gout are underexcretors of uric acid. According to analysis of (NHANES III) hyperuricemia could be resulted from urate over-production due to acquired factors such as high purine diet, fructose ingestion, alcohol intake and decrease with high dairy intake. Fructose ingestion increases urate production by increasing hepatic ATP degradation to AMP to become a urate precursor and rapidly degrade to uric acid (Choi *et al.* 2004 & 2005). In addition, a strong relationship between the ingestion of fructose – containing beverages and both hyperuricemia and gout had been found from several studies (Choi & Curhan, 2008; Gao *et al.* 2007). However, although historically the condition was linked to men's excessive rich foods and alcohol intake, food actually contains only little urate, which is mainly produced in the liver and, to a lesser extent, in the small intestine (Richette & Bardin, 2010). Genetic factors can be the principal contributor to the high prevalence of hyperuricemia in some ethnic groups such as hypoxanthine-guanine phosphoribosyltransferase (HPRT) deficiency and PRPP synthetase superactivity

(Vazquez–Mellado *et al.* 2004).The other contributor factor of increasing serum uric acid concentration are age, sex, body weight, body surface area, body mass and socioeconomic status of an individual (Puig *et al.* 1986). Indeed, hyperuricemia prevalence seems to be increasing worldwide, probably due to longer survival, increased prevalence of hypertension and the usage of certain drugs such as salicylate and pyrazinamide (Kim *et al.* 2003; Vazquez–Mellado *et al.* 2004).

Both metabolic syndrome and type 2 diabetes are more common in patients with gout (Choi *et al.* 2007 & 2008). Furthermore, the hyperuricemia is rises with the occurring of metabolic syndrome and incidence such as hypercholesterolemia, hypertension, obesity, atherosclerosis and impaired carbohydrate metabolism are occurs. The relationship between gout and metabolic syndrome is thought to be mediated by hyperuricaemia whereby serum urate concentrations correlate with the degree of central adiposity and measures of insulin resistance, and insulin inhibits renal tubular uric acid excretion (Facchini *et al.* 1991). In addition, gout and hyperuricemia are also associated with psoriasis and psoriatic arthritis, other emerging factors of cardiovascular risk (Punzi *et al.* 2007; Ramonda *et al.* 2011).

Classically, hyperuricemia is a major risk factor for gout and lithiasis. In addition, it also has been linked to other human diseases such as pre-eclampsia, renal dysfunction, stroke, cardiovascular diseases, hypertension, hyperlipidemia, cancer, diabetes and metabolic syndrome (Vazquez–Mellado *et al.* 2004: Nakagawa *et al.* 2005: Heinig & Johnson, 2006; Choi *et al.* 2007). Further, with an apparent reciprocal relationship between these comorbidities and gout, because their presence may increase the gout flares (Primatesta *et al.* 2011). Generally, hyperuricemia is classified as primary or idiopathic/ genetic and secondary or known as acquired. Both of these types of hyperuricemia occur as a result of increased uric acid production, impaired renal uric acid

excretion or a combination of these mechanisms. For instance, alcohol consumption and obesity are not only associated with increased urate formation but also with reduced renal excretion (Facchini *et al.* 1991). The other condition such as acute or chronic renal failure, less urate is filtered in the glomeruli cause increase of urate levels. Besides this, an enhanced purine turnover rate in Tumor Lysis syndrome which this disease is caused by lymphomas, hematologic malignancies, solid tumors and cytolytic therapy, hemolysis, and psoriasis also can dramatically increase the urate levels (Kalemkerian *et al.* 1997). Nevertheless, the risk of gout and these linked complications are varying depending on the degree of hyperuricemia (Roddy & Doherty, 2010). On the other words, uric acid is sparingly soluble in extracellular body fluids and slight changes in its concentrations may cause supersaturation and precipitation of urate crystals in synovial fluid which leads to attacks of painful acute arthritis (Agudelo, 1989). For this reason, the goal treatment for gout disease is to maintain sustained serum uric acid levels below 6.5 mg/dl in order to prevent acute attacks, tophi and also probably deterioration in renal function. Finally, the control of uric acid level in blood still be considered in the prevention and treatment of gout.

2.4 Pathogenesis of Gout

2.4.1 Clinical Features

Gout is a chronic disease are summarized in three stages: (i) acute arthritis; (ii) intercritical gout; and (iii) chronic tophaceous gout. Acute gouty flare presents with an abrupt onset of severe monoarthritis often at night, red, with extreme pain, warmth, swelling, erythaema that crescendo to maximal intensity over 8–12 hours, fever and, in the elderly, delirium may even be a feature (Michet *et al.* 1995). At first attacks, 90 % are monoarticular, half of which usually involve one joint in the lower limbs, the first metatarsophalangeal joint (podagra). Common sites of acute flares are the feet, ankles, knees and, less commonly, elbows, wrists and fingers. Untreated initial attacks typically subside over 3–10 days but often there are other attacks within 6 months to 2 years. As the inflammation recedes, the overlying skin may exfoliate (Teng *et al.* 2006).

An asymptomatic phase between attacks is called intercritical gout. Despite clinical quiescence, the disease may continue to advance, with variable shortening of intercritical segments as the body urate stores accumulate.

Advanced gout is characterized by chronic destructive arthritis with secondary degenerative changes, bony erosions and development of tophi. Chronic polyarticular gout may be confused with rheumatoid arthritis because of the non–remitting pain, inflammation and deformities associated with tophi, which can be mistaken for rheumatoid nodules. Tophi development is a function of early age of onset, long duration of untreated disease and high serum urate levels. However, tophies are found most commonly in the finger pads, wrists, pinnae, knees and olecranon bursae, and over pressure points, such as extensor aspect of the forearm and Achilles' tendon. In some patients, chronic tophaceous gout may develop with progressive joint destruction and

disability or destructive arthropathy (Choi *et al.* 2005; Nuki & Simkin, 2006; Richette & Bardin, 2010: Marson & Pasero, 2012).

2.4.2 Pathophysiology of Acute Inflammation Gout, Chronic Tophaceous Gout and Resolution Phase

The presence of monosodium urate crystals (MSU) in synovial fluid or tophi is main criterion for diagnosis of gout (Weselman & Agudelo, 2001). It could be said that a number of endogenous substances and physicochemical conditions affect crystal precipitation, growth and even dissolution, regulating not only MSU crystal metabolism but also their inflammatory activity (Oliviero *et al.* 2011). Besides that, the presence of certain protein including, immunoglobulin (particularly IgM) and insoluble collagen associate with the accumulation of debris within the synovial cavity may provide a nucleus for crystal development (Kanevets *et al.* 2009).

The sequel of formation of monosodium urate (MSU) crystals resulting from regulation of innate immunity response is summarized in Figure 2.4. Phagocytosis of MSU crystals is greatly promoted by opsonization by IgG or complement components (Dalbeth, 2005). MSU crystals cause a massive of neutrophil influx into the synovium and joint fluid, where they phagocytise crystals actively (Haskard & Landis, 2002). The consequences of neutrophil interaction with MSU crystals and phagocytosis caused the release of a large variety of mediators that promote the vasodilatation, erythema and pain associated with the acute gout attack. These include reactive oxygen species such as superoxide, hydrogen peroxide and singlet oxygen, nitric oxide, leukotriene B4, prostaglandin E2, anti-microbial peptides, enzymes, IL $-$ 1, and chemokines such as S100A8, S100A9 and IL$-$ 8 (Dalbeth, 2005). MSU that have undergone phagocytosis activate the NLRP3 inflammasome, leading to secretion of interleukin $-$ 1β while

neutrophil ingress by crystals is therefore crucial to the induction and perpetuation of the inflammatory response.

The interaction of innate immunity response in acute gouty inflammation was reinforced by activation of cytoplasmic NACHT−LRRPYD-containing protein−3 (NALP3), also known as cryopyrin, inflammasoma complex is crucial for acute MSU crystal-induced inflamamation. In fact, the releasing of mature interleukin, IL−1β, by stimulation of MSU crystals requires activation of NLRP3 inflammasome: an intracellular protein complex composed of a member of the NALP protein family and the adaptor protein ASC that connects the NALPs with caspase−1. Triggering of NLRP3 leads to activation of caspase−1, which cleaves pro-IL−1β to the active secreted product (Figure 2.5). Furthermore, macrophages are prerequisite for components of the NLRP3 inflammasome able to secrete active IL − 1β following triggering with MSU crystals (Martinon *et al.* 2006).

In addition, caspase − 1 activation, IL − 1ß processing and release and triggering of inflammation are driven by link TLR (TLR2 and TLR4 adaptor molecule) − mediated recognition events from CD4 for MSU crystals with intracellular engagement of the crystal by the NALP3 inflammasome (Scott *et al.* 2006). The other component such as leucine-rich repeat (LRR) domain of cryopyrin also required for MSU crystal−induced inflammatory response (Hoffman *et al.* 2010). Despite the role of biological cellular pathways in acute gout attack, IL−1β is a crucial mediator for gout and MSU crystal− induced inflammation. (Terkeltaub *et al.* 2009).

Chiefly, the acute gouty arthritis involved high levels of cytokines such as IL − 1β, IL − 6, TNF − α and chemokines such as IL − 8 and CCL2 in synovial fluid (McNearney *et al.* 2004; Scanu *et al.* 2012). As shown in Figure 2.5, phagolysosomes, are produced when crystals initially lie within phagosomes once received the hydrolases

by degranulation of lysosomes. The substances coating the urate crystals are then digested by the hydrolases, and the crystals come in direct contact with the phagolysosomal membrane and form hydrogen bonds. The phagolysosomal membrane is damaged and the contents, including the hydrolases, leak out into the free cytoplasm and to the extracellular media injuring the host cell and eventually lead to cell disruption (Hoffstein & Weissmann, 1975). The MSU crystals thus released are again ingested by other cells which in turn degranulate and die, thus possibly recycling the above series of events.

Figure 2.5 showed that after pro-IL−1β stimulated by MSU crystals, the other components also contribute in modulating the process of IL−1β maturation and released which could be mediated by a second signal such as extracellular ATP through its receptor P2X7 and potassium (K^+) efflux or reactive oxygen species (ROS) (Piccini *et al.* & Cassel *et al.* 2008).

In chronic gout, the presence of prolong hyperuricemia cause some patients develop tophi within joints and soft tissues. These lesions response depict a foreign body granulomatous response to MSU crystals and may invade bone caused bone erosion and joint damage with low-grade synovitis and frequently recurring or non-resolving flares, these inflammatory processes are probably ongoing with potentially continued release of inflammatory mediators, including interleukin−1β, in the presence of persistent monosodium urate crystals (Neogi, 2011).
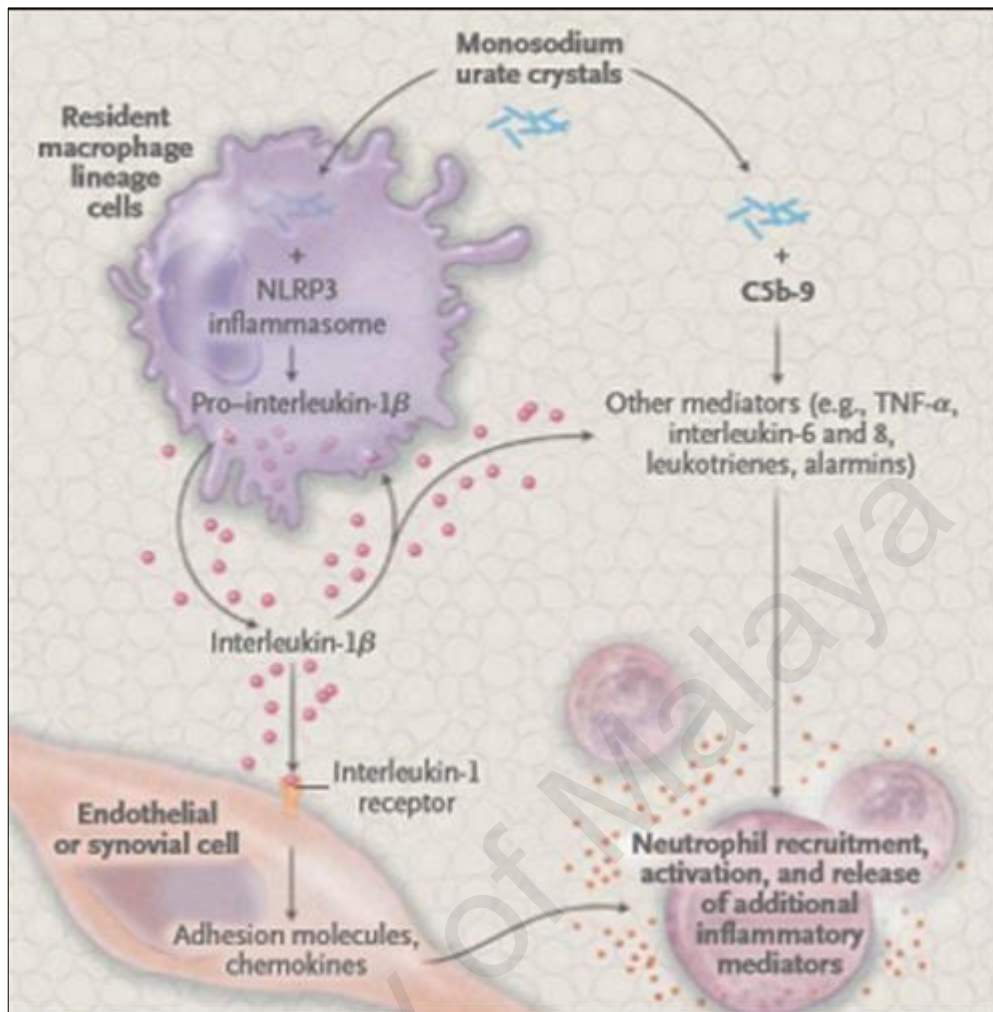
Figure 2.4. Sequel of formation of monosodium urate crystals (MSU) in gout in innate immunity. Adapted from Neogi, (2011).
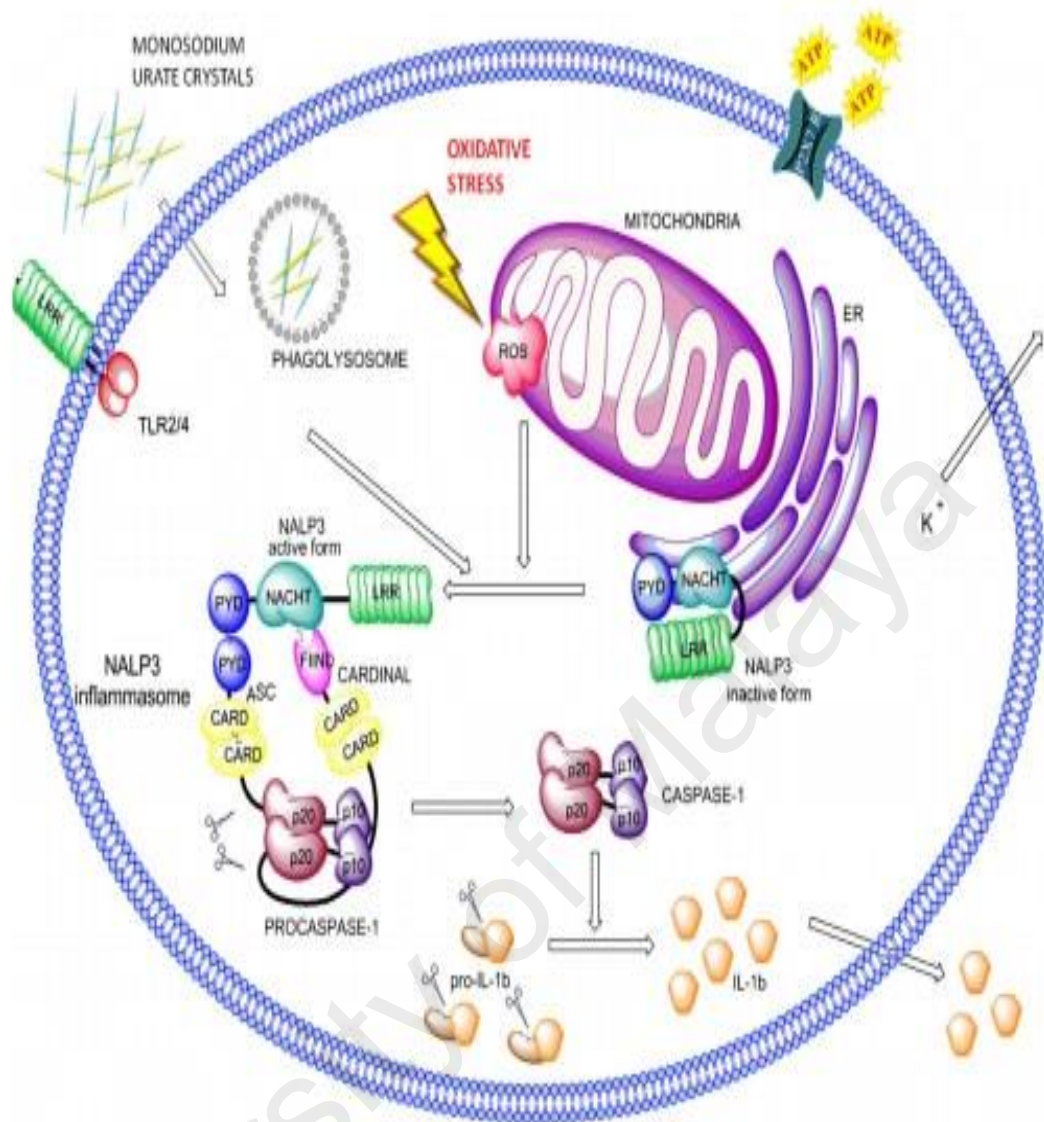
Figure 2.5. Inflammasome activation induced by monosodium urate crystals. MSU crystals, once recognized and phagocytosed by cells, interact with the innate immunity receptor NALP3 leading to its activation. Consequently, caspase-1 cleaves the inactive pro-IL–1ß to the active IL–1ß. ATP accumulation and reactive oxygen species (ROS) production induced by crystals, also contribute to NALP3 activation. Adapted from Punzi *et al.* (2012).

2.4.3. Treatment

The primary goal of gout management is to reduce serum urate to target levels < 6 mg/dl through lifestyle modification and use of pharmacologic urate–lowering therapies (ULT) including xanthine oxidase inhibitors, uricosuric agents, and uricase agent. Unfortunately, ULT may increase the risk of gout flares and, gout management guidelines recommend concomitant used of colchicine and non-steroidal anti-inflammatory drugs (NSAID) for prophylaxis of ULT– associated gout flares (Khanna *et al.* 2012). However, these prophylactic agents are associated with well-recognized side effects, and the presence of the specific comorbid conditions, especially in an older population, and those with heart failure, renal insufficiency, hepatic dysfunction, or known gastrointestinal symptoms which these contraindications may pose barriers to their use (Spieker *et al.* 2002; Hoskison & Wortmann, 2007; Keenan *et al.* 2011; Neogi, 2011).

The interaction between the gout attack and the comorbid conditions make the management and treatment of gout more complex, and suggest the need for alternative medicine which derived from natural source that have a more favorable safety and tolerability profile in patients. Xanthine oxidase (XO) inhibitors, including allopurinol and febuxostat block the synthesis of uric acid and can be used regardless of whether there is overproduction of urate. At present, allopurinol is the type of XO inhibitor drug that commonly used in clinical application of hyperuricemia or particularly among patients in whom target urate levels are achieved and gout which is effective in decreasing flares and tophi (Becker *et al.* 2005; Becker *et al.* 2010). The other class which is uricosuric drugs including probenecid, block renal tubular urate reabsorption. Although these drugs can be used in patients with underexcretion of urate which is predisposing for up to 90 % of patients with gout, they are used less frequently than XO inhibitors or allopurinol and are contraindicated in patients with a history of nephrolithiasis (Reinders *et al.* 2009). Uricase including pegloticase, converts uric acid into soluble allantoin. This

class of drug is use for chronic gout in adults whose disease is refractory to conventional therapy; e.g., lack of normalization of serum urate, inadequate control of signs and symptoms with the use of a XO inhibitor at maximum medically appropriate dose, or other contraindication. The other consideration of this drug is a risk of infusion reactions (26 %, vs. 5 % in placebo group) even with premedication, particularly in patients without a therapeutic response (in whom serum urate levels increase to above 6 mg per deciliter, particularly on two consecutive occasions) or with antibodies against pegloticase. Anaphylaxis occurs in 5 % of patients (vs. 0 % in placebo group).In addition, cost is higher than for other drugs (Sundy, 2008).

Understanding all the mentioned of contributed factors and the complicated mechanism pathway for gout will help us in identifying what type of gout we are targeting for the treatment as the treatment of this disease depends on the type of attack. NSAIDs and colchicine are first-line agents for acute gouty attacks. Meanwhile, controlling the uric acid level in the blood is still the main therapy particularly in the management of the chronic gout attacks especially allopurinol (Mandell, 2002).

2.5 Reactive Oxygen Species and Gout

ROS are defined as an atoms or small molecules that have unpaired valence shell electrons. They readily accept another electron or transfer their unpaired electron to another molecule. ROS are formed as by-products of basal cellular metabolism as shown in Figure 2.6. Under normal conditions, approximately 95 % of molecular oxygen in biological systems undergoes controlled reduction through the addition of four electrons (tetravalent) in the mitochondrial cytochrome oxidase system to form water. The remaining molecular oxygen undergoes sequential, univalent reduction to produce partially reduced oxygen free radical intermediates, superoxide anion, hydrogen peroxide and the highly reactive hydroxyl radical. However, alterations in the amount and the nature of released of ROS occurs in various disease state (Halliwell & Gutteridge, 1990).
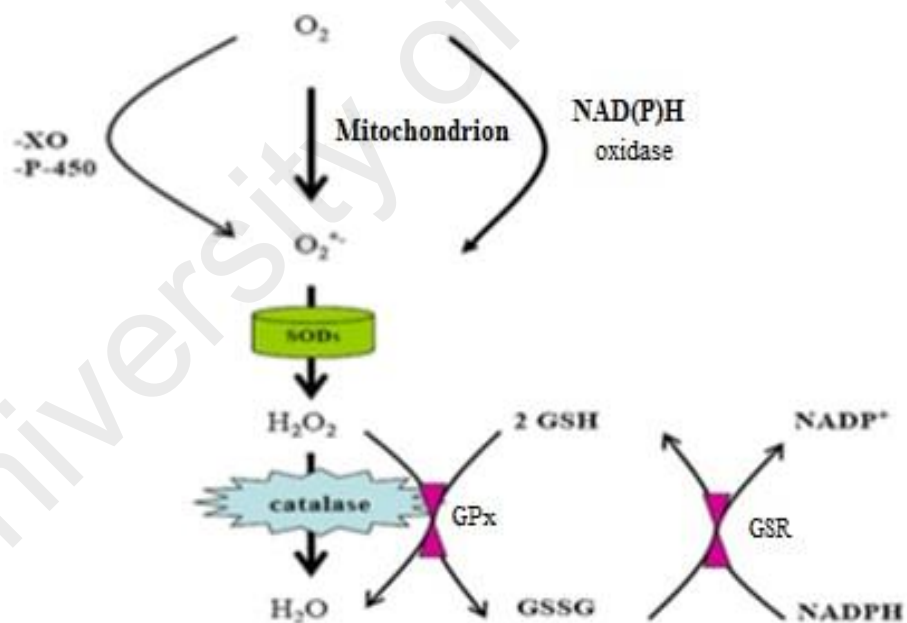
Figure 2.6. Pathways for the production and removal of reactive oxygen species. GSH, glutathione; GSSG, oxidized glutathione; GSR, glutathione reductase; XO, xanthine oxidase. Adapted from Afonso *et al.* (2007).

Reactivity varies widely from one ROS to another's. Among ROS produced by living cells, superoxide anion, $O_2{}^-$ is a proinflammatory compound that damages cells and the ECM. Figure 2.7 shown the subsidiary product of superoxide anion, $O_2{}^{\cdot-}$ in living organism. $O_2{}^{\cdot-}$ capable to damages endothelial cells and increasing the permeability of the microvasculature then promoting the migration of neutrophils to foci of inflammation. $O_2{}^{\cdot-}$ can be converted to other, more aggressive, ROS such as the hydroxyl radical $HO\cdot$, which is produced when $O_2{}^{\cdot-}$ interacts with free iron or copper ions. These ions are normally present in tiny amounts in healthy individuals, as they are sequestered by specialized proteins such as ferritin, so that virtually no $HO\cdot$ is produced. $O_2{}^{\cdot-}$ also can combine with NO to produce peroxynitrite ($ONOO^-$), thereby modifying the bioavailability of NO. Hydrogen peroxide (H_2O_2) can diffuse in the cytoplasm and cross cell membranes. Some ROS act as mediators that regulate cell functions such as proliferation and apoptosis, by influencing intracellular signaling pathways via effects on gene expression (Haddad, 2002).
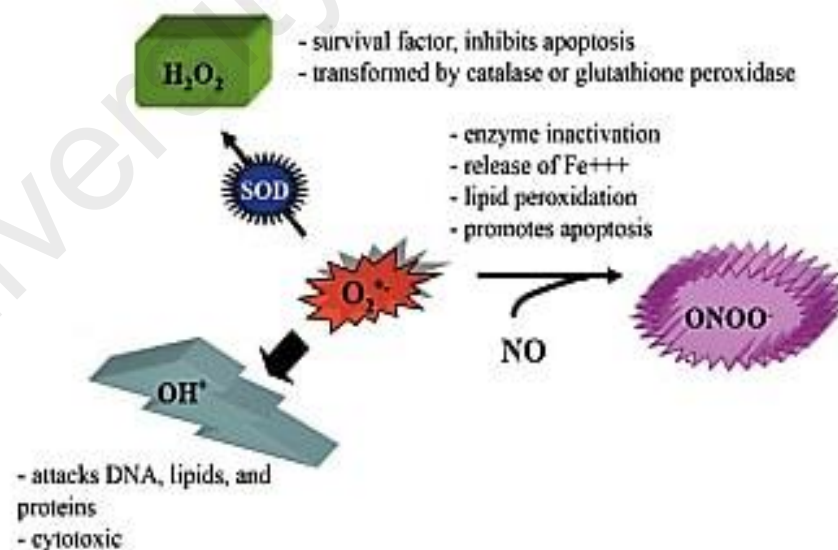
Figure 2.7. Superoxide anion ($O_2{}^{\cdot-}$) and its derivatives. Adapted from Afonso *et al.* (2007).

From the Figure 2.6, endothelial xanthine oxidase could contributed to ROS production. Xanthine Oxidase, (XO) is main sources of ROS during purine biosynthesis pathway which attributable to production of uric acid and thus, become a target of therapeutic drugs against hyperuricemia or gout (Elion, 1989). XO, an isoform of xanthine oxidorreductase (XOR), an enzyme that catalyses the final steps in the purines metabolism (Parks & Granger, 1986). XOR exists in two interconvertible forms, either as xanthine dehydrogenase (XDH), which predominates in healthy tissues, and as XO, which appears to have an important role in cell and tissue injuries (Harrison, 2002). Both forms catalyse the conversion of hypoxanthine to xanthine and then to uric acid, which is final reactions in the metabolism of purine bases however, the XDH can reduce either oxygen or NAD^+ and generates the reduced β – nicotinamide adenine dinucleotide (NADH), whereas XO reduces molecular oxygen, leading to the production of ROS, such as hydrogen peroxide (H_2O_2) and superoxide anion (Berry & Hare 2004). The dual role of XO means that it is an important regulator of cellular redox state (Elahi *et al.* 2009). The accumulation of uric acid in the body is responsible for several diseases and thus it plays a vital role in hyperuricemia and gout. This, mechanism yielding superoxide radicals and raises the oxidative level in an organism. During the reoxidation of xanthine oxidase, molecular oxygen acts as electron acceptor, producing superoxide radical and hydrogen peroxide. Hydroxylation takes place at the molybdopterin center (Mo–pt) via Mo–OH oxygen which forms a bond with a carbon atom of the substrate such that the oxygen atom is derived from water rather than molecular oxygen (Okamoto *et al.*, 2004). Thus, treatment with XO inhibitor are found to prevent the development of various disease including endothelial dysfunction, atherosclerosis, gout and given during initiation of chemotherapy in myeloproliferative malignancies, in order to prevent the harmful effects of high production of uric acid (Schroder *et al.* 2006).

2.6 Xanthine Oxidase

Xanthine oxidoreductase (XOR), or also known as Xanthine Oxidase (XO) a member of the molybdenum hydroxylase flavoprotein family which also a complex enzyme long known to be present in the bovine milk fat globule membrane (Patton & Keenan, 1975). XO is the most well documented biological source of reactive oxygen species and has been detected in a large number of bacteria which comprising of at least eight phyla and innumerable classes of the animal kingdom. The highest xanthine oxidase activity had been found at liver and small intestine from the mammalian tissue studied, (Parks & Granger, 1986). In humans, apart from liver and intestine, most tissues show little XO activity (Sarnesto *et al.*, 1996). XO was purified and characterized sucesfully from various species including the human breast in 1986 (Krenitsky *et al.* 1986). Regulation of XO activity is important during inflammation (Nakai *et al.* 2006). XO has high amino acids sequence homology among the mouse, rat, bovine and human enzymes with about 90 % identity, and consisting of about 1,330 amino acids (Nishino, 1994; Borges *et al.* 2002; Godber *et al.* 2005). Studies showed the existence of similar physicochemical properties of XO between human and bovine milk, but it has only approximately 5 % of the activity of the bovine milk enzyme towards xanthine and related substrates proposing that the low activity is due to grossly deficient of molybdenum (Abadeh *et al.* 1992).

The crystal structure is a homodimer of 150 kDa subunits (Enroth *et al.* 2000). Each of the subunits is composed of three domains, as shown in homodimer structure in Figure 2.8. The right side is cofactor arrangement of XO. The N-terminal (in red), the C-terminal (in blue) and the intermediate (in yellow) domains contain the iron-sulfur centers, the molybdopterin and the FAD centers. The largest domain contains the molybdenum center (molybdenum cofactor; molybdopterin), the intermediate contains flavin adenine dinucleotide (FAD) cofactor and the smallest contains the two iron sulfur

27

centers ([2Fe–2S] type). The redox reaction centers are almost linearly positioned in the order of molybdopterin, two [2Fe–2S] type iron sulfur centers and FAD. The iron sulfur centers are called Fe/S I and Fe/S II, based on their redox potentials and EPR signals (Iwasaki *et al.* 2000; Caldeira *et al.* 2000). Fe/S II has the higher redox potential. Electrons that are passed to the molybdenum during the hydroxylation reaction are transferred to FAD via the iron sulfur centers. Finally, NAD^+ or oxygen molecule, which is the final electron acceptor, is reduced (Okamoto *et al.*, 2013). Arrows in Figure 2.8 show the directions of electron flow during catalysis. The reduced FAD reacts with either NAD^+ or oxygen to produce NADH or hydrogen peroxide (H_2O_2) or superoxide (O_2^-). $FADH_2$ reacts with O_2 to produce H_2O_2, while FADH produces O_2.
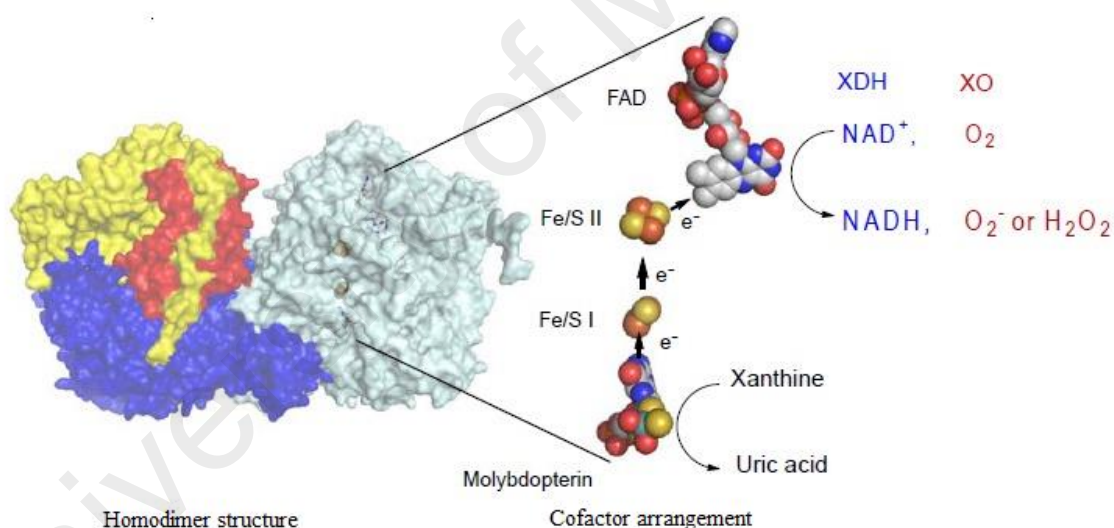
Figure 2.8. Crystal structure of bovine milk from Protein Data Bank (entry code 1F4Q). Adapted from Nishino & Okamoto, (2015)

The crystal structures of bovine milk XO as shown in Figure 2.8, suggests that hypoxanthine and xanthine bind to the C–terminal domain of the XO, close to the molybdopterin cofactor. During substrate oxidation, the molybdenum center (Mo centre) is first reduced by electron received from the substrate and subsequently re–oxidized, as

the electron passes first to the iron- sulfur centres and then to the FAD centre, and are finally donated to NAD^+ or O_2 (Ratnam, 1995; Kisker *et al.* 1998; McMaster & Enemark, 1998). Salicylate was crystallised with XO as a competitive inhibitor, and it bound to the Mo active site. Although salicylate itself does not bind to the Mo−pt cofactor, it blocks the approach of substrates toward the metal complex. Several amino acid residues, including Arg 880, Phe 914, Phe 1009, Thr 1010, and Glu 1261 (Figure 2.9) contributed important hydrogen and electostatic interactions between salicylate and the binding site. The salicylate molecule is kept in place by several hydrogen bonds and electrostatic interactions.
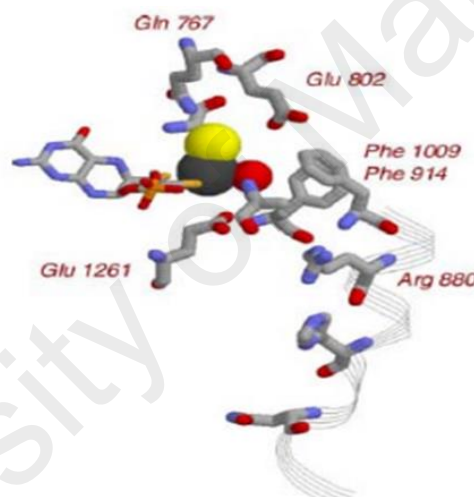
Figure 2.9. Amino acid residues involved in the catalytic reaction in the Mo-pt active site of XO. Adapted from Yamaguchi *et al.* (2007).

Two catalytic reactions are involved in mechanism of XO; reductive half and the oxidative half reaction. The reductive half reaction is takes place in the molybdenum center of the enzyme while Mo(VI) is converted to its reduced form Mo(IV) which contributed to conversion of xanthine to uric acid. While the oxidative half reaction takes place at the FAD center, and oxidize Mo(IV) to Mo(VI) by molecular oxygen with the formation of ($O^•_2{}^-$) or H_2O_2 (Huber *et al.* 1996).

The oxidative part of the reaction involves the binding of molecular oxygen to the FAD centre of the reduced form of XO. This will lead to the transfer of electrons from the reduced form, to achieve enzyme oxidation (Harris & Massey, 1997) as shown in Scheme 2.1. Slow and fast phase in this reaction have been observed, and six electrons are transferred throughout the reaction (Mondal *et al.* 2000) as shown in Scheme 2.2. The fast phase involves in the transfer of five electrons and the formation of hydrogen peroxide (H_2O_2) and superoxide ($O_2^{.-}$). Whereas the slow phase includes the oxidation of one electron and the formation of superoxide only (Mondal & Mitra, 1996) as given in Scheme 2.3. The mechanism of the formation of xanthine and uric acid is considered as the reductive half reaction (Scheme 2.4) (Kim *et al.* 1996).

$$(XO)_{reduced} + O_2 \rightleftharpoons (XO)_{reduced} \cdot O_2 \longrightarrow (XO)_{oxidized} + H_2O_2$$

Scheme 2.1

$$XO(6e) \longrightarrow XO(4e) \longrightarrow XO(2e) \longrightarrow XO(1e) \longrightarrow XO(0e)$$

Scheme 2.2

Fast phase:

$$XO(6e) + O_2^{.} \longrightarrow XO(6e) \cdot O_2 \longrightarrow XO(1e) + O_2^{.-} + H_2O_2$$

Slow phase:

$$XO(1e) + O_2 \longrightarrow XO(0e) + O_2^{.-}$$

Scheme 2.3

$$(XO)_{oxidized} + Xanthine \rightleftharpoons Xanthine - (XO)_{oxidized}$$
$$\downarrow$$
$$(XO)_{reduced} + Uric\ acid$$
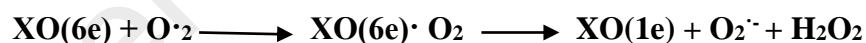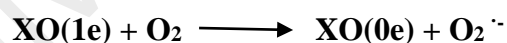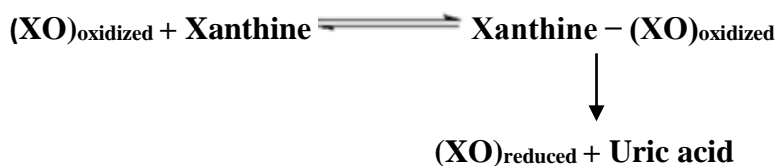
Scheme 2.4

2.7 Role of medicinal plants and natural products in gout

Natural products have been contributed extensively towards the development of modern medicine, and continuously play a significant role in drug discovery. South-east Asian countries like Malaysia, Thailand and Borneo have a long history of using medicinal plant that offer considerable pharmaceutical potential. Malaysia houses more than 8,000 species of flowering plants, including shrubs, herbs, lianas, and epiphytes holds great promise for the discovery of novel biologically-active compound (Aziz *et al.* 2011). Out of the total flowering plants reported from the world, more than 50,000 are used for medicinal purposes (Teklehaymanot & Giday, 2007).

Natural products discovered from medicinal plants have provided numerous clinical useful medicines and can be predicted to remain an essential component in the search for new medicines (Balunas & Kinghorn, 2005). Because current treatments for gout result in undesirable side effects and tend to be expensive, natural products devoid of such disadvantages offer a great opportunity. Since ancient times, plant-based medicines which in form of dispensed in the form of extracts, tinctures, powders or poultices have been used for the alleviation of pain and other inflammatory conditions including gout. In India and Pakistan, healers use *Coccinia grandis*, *Datura metel*, *Stychnosnux vomica*, *Pistacia integerrima* and *Vitex negundo*. Anti−inflammatory and gout therapy is dominated by opioids and non-steroidal anti−inflammatory drugs (NSAIDs) but both classes of drugs produce serious side effects. The search for pharmacological agents to overcome these shortcomings has become a major goal in pain research. Recently, their XO−inhibitory effects have been elucidated as the mechanism for their anti-gout effects (Umamaheswari *et al.* 2007; Ahmad *et al.* 2008). Scientists have turned to explore the potent XO inhibitor from a wide variety of traditional folk medicines. Natural XO inhibitors from *in vitro* studies were reported from a variety of

plants used as traditional herbal medicines in different countries in the world such as *Coccinia grandis* and *Vitex negundo* in India (Umamaheswari *et al.* 2007), *Chrysanthemum sinense* and *Tetracera scandens* in Vietnam (Nguyen *et al.* 2004), *Cleodendrum floribundum*, *Eremophila maculata* and *Stemodia grossa* in Australia (Sweeney *et al.* 2001), *Cinnamomum cassia*, *Chrysanthemum indicum* and *Lycopus europaeus* and *Polygonum cuspidatum* in China (Kong *et al.* 2000a), *Larix laricina* in North America (Owen & Johns, 1999), *Hyptis obtusiflora* and *Hyptis lantanaefolia* in Panama (Gonzalez *et al.* 1995) and *Hexachlamys edulis* and *Eugenia punicifolia* in Paraguay (Theduloz *et al.* 1988) and Polynesian traditional fruit, *Morinda citrifolia* (Palu *et al.* 2009). In general, the methanol extracts were found to be more active than the methanol-water or water extracts (Kong *et al.* 2000a; Nguyen *et al.* 2004).

2.8 Studied Plant- *Plumeria rubra* Linn

2.8.1 Taxonomy of *Plumeria rubra* Linn.

Kingdom- Plantae

Class- Magnoliopsida

Subclass- Asteridae

Order- Gentianales

Family- Apocynaceae

Genus- *Plumeria*

Species- *rubra*

Figure 2.10. *Plumeria rubra* tree

Plumeria rubra Linn which belong to family Apocynaceae is kind of shrub tree which growing in tropical and sub-tropical regions of the world and distributed from Southern Mexico to Southest Asia (Ye *et al.* 2009). In Malaysia this famous garden plant is commonly known as 'kemboja,' and several other names such as 'pokok kubur' and 'bunga kubur' (Comer, 1997).

The tree is deciduous and has very thick, stout branches with milky latex. The leaves are spirally arranged near the ends of the swollen branches. They are recognized as excellent ornamental plants and often seen in the graveyards. It grows as a spreading tree with crooked trunk and rough bark, swollen branches leafy at the tips, elongated alternate leaves (Pino *et al.* 1994). The shape of the leaf can be described as elliptical, oblanceolate and rarely lanceolate, while the tip is described as acuminate, acute or obtuse (Chinn, 1986). The flowers are generally large, waxy and very fragrant, in terminal or lateral stalked clusters (Burkill, 1966). This gorgeous tropical tree consist of reddish blooms and spiral–shaped which appears at the branch tips is commonly used in decoration such as leis and wreaths (Ye *et al.* 2009). *Plumeria* plants are famous for their attractiveness and fragrant flower due to delightful aroma which is most intense during the early part of the day.

Figure 2.11. *Plumeria rubra* **(A) Leaves (B) Flowers**
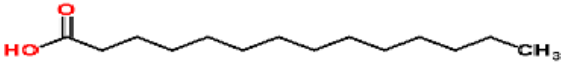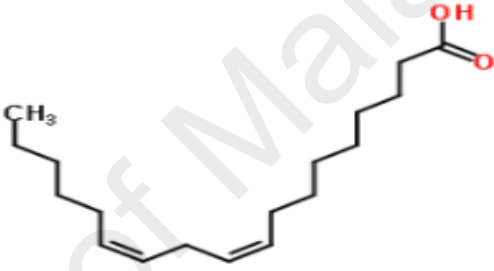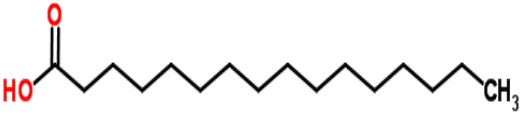
2.8.2 Traditional and Medicinal Used of *Plumeria rubra* Linnn

There are numerous reports on the ethnomedical uses of plants belonging to the pantropic genus *Plumeria* (Hamburger *et al.* 1991). Traditionally, the decoction of the bark is used as a purgative. emmenagogue, febrifuge or diuretic, and to cure gonorrhea, dropsy and dysuria due to venereal disease, while the decoction of the leaves is used as a lotion for cracks and eruptions on the soles of the feet (Lim, 2011). The leaves of *P.rubra* L. are used in ulcers, leprosy, inflammations and rubefacient (Bobbarala *et al.* 2009; Gupta *et al.* 2006). Wiart, (2002) reported that the decoction of the bark and roots of *P.rubra* L. is traditionally used to treat asthma, ease constipation, promote menstruation and reduce fever whereas the latex is used to soothe irritation. The fruit is reported to be eaten in West Indies. In India, however, it has been used as an abortifacient (Zaheer *et al.* 2010). In Indonesia, the plant is cultivated as an ornamental species and decoctions prepared from the bark is used to treat venereal diseases (Burkill, 1966). The roots and rhizomes of this plant are also used against snakebite (Houghton & Osibogun, 1993). The essential oils from the flowers are used for perfumery and aromatherapy purposes (Wiart, 2002). The Siamese use an infusion of the flowers in cosmetics, applied after bathing (Burkill, 1966). The flowers are aromatic and widely used in pectoral syrups. The flowers decoction of *P. rubra* was reported to be used in Mexico for control of diabetes mellitus (Bobbarala *et al.* 2009). In addition, *P.rubra* L. flowers are known to contain potent cardioactive glycosides that have positive inotropic effects on the heart (Frishman *et al.* 2009). Furthermore, *P.rubra* L. is also has been reported that among the traditional plants claimed to exhibit anti-tumoural, antimicrobial and cytotoxic activities and is also effective against parasitic infestations, such as scabies and pediculosis (Monzon, 1995; Nargis *et al.* 1994).

2.8.3 Chemical Constituents of *Plumeria rubra* Linn

Phytochemical studies of the flowers of *P. rubra* L., are found to contain tannins, flavonoids, terpenoids, reducing sugars and alkaloids.

Table 2.1 Chemical Compounds found in *P.rubra* L.

Phytochemical compounds	Chemical Structure
Myristic acid	Tohar *et al.* 2006
Linoleic acid	Tohar *et al.* 2006
Lauric acid	Tohar *et al.* 2006
Palmitic acid	Tohar *et al.* 2006

Plumericidine
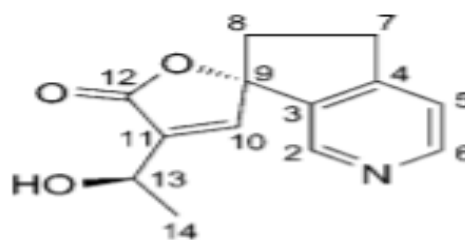
Ye *et al.* 2009

Anthocyanin

1. cyaniding–3–O–β–(2''–
glucopyranosyl–O–β–
galactopyranoside)
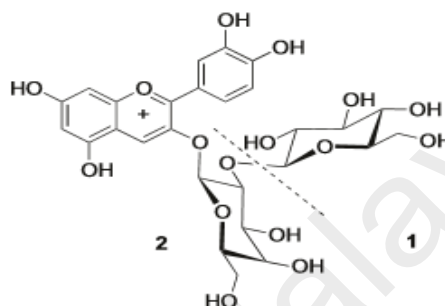
2.cyanidin–3–O–β–
galactopyranoside

Byamukama *et al.* 2011

Rubranonoside (=7–O–α–L–
rhamnopyranosyl–4'–O–β–
D –
glucopyranosylnaringenin
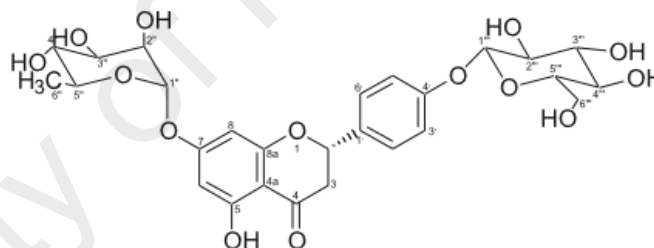
Akhtar *et al.* 2013

Rubranin   (new
flavanone glycoside)
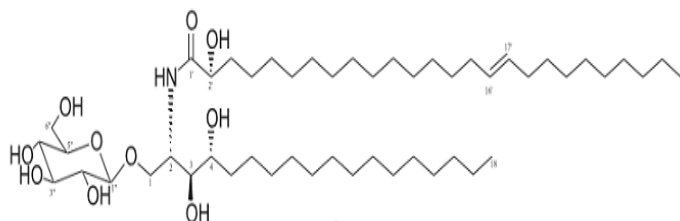
Akhtar *et al.* 2013

Rubradoid (plumieridine –
1 – O – β – D –
galactopyranoside)
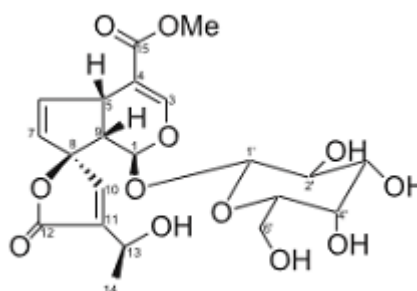
Akhtar *et al.* 2013

Rubrajaleelol (new iridoid galactoside)
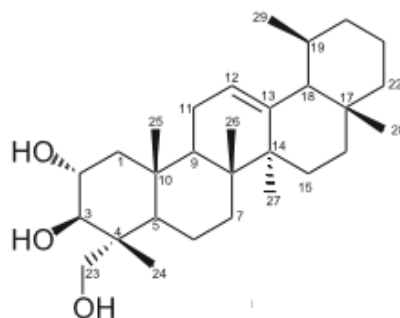
Akhtar *et al.* 2013

Rubrajaleelic acid
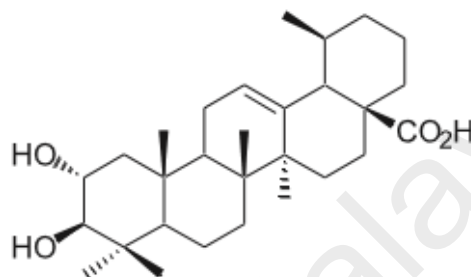
Akhtar *et al.* 2013

1−α−plumieride (two new nor−terpenoids with known iridoids)
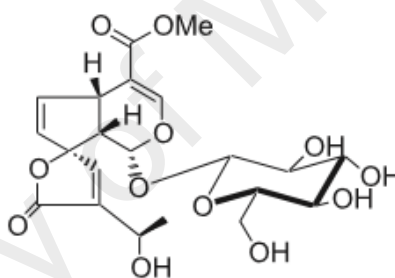
Akhtar *et al.* 2013

Plumieride p−z−coumarate
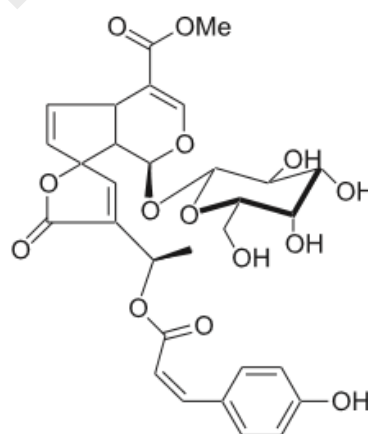
Akhtar *et al.* 2013

2.9. Phytochemical Compounds with Xanthine Oxidase Inhibitor

Some of herbal plants and their phytochemicals are worth to be explored as potential xanthine oxidase inhibitor as they are already used as food or food supplements for many years and found to be safe for human bodies (Azmi *et al.* 2012). More often, plant based-natural products provide lead structures which are starting points for chemical modification to derive an optimal drug (Bezerra *et al.* 2013). In the past two decades, the potential of free radical scavenging and XO inhibitors were explored from a wide variety of traditionally used herbal plants (Montoro *et al.* 2005). Thus, most research on medicinal plants focused on their active chemical constituents currently used and those with the potential to be developed as anti–gout medications in the future.

The anti–gout effects of these medicinal plants include three mechanisms which including (i) the decrease of uric acid production, (ii) uricosuric effects and (iii) anti-inflammatory activities. Under the three main mechanisms of pathogenesis, medicinal plants that can be potent in the treatment of gout are collected and described, together with their active phytochemicals. Ten classes of compounds derived from plants with anti-gout potential had been identified. Flavonoid which are very potent anti-gout mediators due to their multiple effects correlated with different mechanisms, including genistein, astilbin, apigenin, quercetin, myricetin, liquiritigenin, isoliquiritigenin, rutin, procyanidin, luteolin and apigenin–4'–O–(2–O–p–coumaroyl)–d–glucopyranoside. Flavonoid exhibit *in vitro*, *in vivo*, or both of the two XO inhibitory effect even with different inhibition modes (Gariboldi *et al.* 1998; Kong *et al.* 2000; Jiao *et al.* 2006; Cimanga *et al.* 2010; Chen *et al.* 2011; Huang *et al.* 2011). Morin, rutin and quercetin revealed both XO inhibitory activity and uricosuric action (Yu *et al.* 2006; Shi *et al.* 2012). Luteolin and apigenin give anti-inflammtory effect (de Souza *et al.* 2012). Caffeic acid, luteolin, eriodictyol and 1, 5–di-O–caffeoylquinic acid from *Chrysanthemum sinense* have demonstrated significant XO inhibition (Nguyen *et al.* 2004). The other studies

which also revealed good XO inhibitory activity were including water –soluble phenolics and tannin (Owen & Johns, 1999), resveratrol (Chen *et al.* 2011) and melanoxetin and okanin (Tung & Chang, 2010). Both resveratrol and lithospermic acid give not only XO inhibitory activity but also anti–inflammatory effects (Soung *et al.* 2003; Aggarwal *et al.* 2004; Shishodia & Aggarwal, 2006; Liu *et al.* 2008). Recently, chlorogenic acid, which is the one of the most abundant polyphenols in the Chinese medicines, has been reported to have an anti–gout effect. As less literature reported that chlorogenic acid was known phytochemical that acts as XO inhibitors but surprisingly Meng *et al.* (2014) has been found that chlorogenic acid could decreased the serum uric acid level by inhibiting the xanthine oxidase (XOD) activity but not increasing the urinary uric acid level in potassium oxonate (PO)–induced hyperuricemia of mice model. In addition, chlorogenic acid also exhibited the effect of suppressing paw swelling of MSU crystal-induced inflammation in rat. Tannin compounds like geraniin, corilagin and gallic acid which isolated from *Geranium sibiricum* Linn had elicited uricosuric action (Wu *et al.* 2010) while ellagic acid which isolated from longan had suppressed XO activity (Hou *et al.* 2012). Berberine is a potent XO inhibitor (Kong *et al.* 2004), while other alkaloids, colchicine and piperine give very good anti-inflammatory activity (Ahern *et al.* 1987; Sabina *et al.* 2011). Of note, colchicine is a classical anti-gout drug with effects of both pain relief and inflammation resistance. Additionally, the crude extract of *Jatropha isabellei* that is rich of alkaloids also presents antinociceptive and anti-inflammatory effects (Silva *et al.* 2013). An essential oil component, cinnamaldehyde, shows dual urate lowering effects, XO inhibitory and uricosuric action (Wang *et al.* 2008). The later effect is hypouricemic action activating the blocked urate transporter (UAT) activity induced by oxonate in the hyperuricemia model of mice (Zhao *et al.* 2006). An iridoid glucoside, specioside and two coumarins, fraxetin and esculetin reveal XO inhibitory (Kong *et al.* 2000; Kumar *et al.* 2005). Lupeol, a triterpenoid, exerst anti-inflammatory effects (de

Souza *et al.* 2012). The lignans, arctigenin from *Arctium lappa* L., exert anti-inflammatory activities through inhibition of the iNOS pathway (Zhao *et al.* 2009), other three lignans, phyllanthin, hypophyllanthin and phyltetralin of *Phyllanthus niruri* exhibit both XO inhibitory and uricosuric action (Murugaiyah & Chan 2009). The only phytochemical compound isolated from phycophyta in this review is fucoxanthin, a kind of xanthophyll, possesses potential anti-inflammatory activity (Heo *et al.* 2010).

Studies by Vivot *et al.* (2001); Kumar *et al.* (2005); Boumerfeg *et al.* (2009); Wu *et al.* (2010) revealed that the plants which give antioxidant activities should be highly potent in the management of gout because they often share XO inhibitory effects. Furthermore, it is generally believed that plants with higher phenolic content show good antioxidant activity. In other words, there is a direct correlation between total phenol contents and antioxidant activity (Brighente *et al.* 2007; Biglari *et al.* 2008; Salazar *et al.* 2008). Thus it can be stated that the phenolic content of the plant may be a good indicator of its antioxidant capacity (Chanda & Nagani, 2010), and even of its potential to be used in anti-gout treatment.

CHAPTER 3

METHODOLOGY

3.1 Plant Materials

The red flowers of *P.rubra* were collected in February, 2014 in area of Faculty Science, University of Malaya. The materials were identified by Prof. Dr Ong Hean Choi from Institute Biological Science, University of Malaya. A voucher specimen; reference no: KLU 48177 was deposited in the Rimba Ilmu herbarium, University Malaya.

3.2 Instruments and Chemical & Reagents

ELISA microplate reader Tecan Sunrise (Austria), LCMS Flexar FX−15 UHPLC, USA, Spectrophotometry UV − 1700 Shimadzu, Japan, 2,2diphenyl − 1 − picrylhydrazyl (DPPH), 2,4,6−tripyridyl−s−triazine (TPTZ), gallic acid monohydrate, ferrozine, sodium nitroferricyanide(III)dehydrate, hydrogen peroxide, sodium hydroxide, aluminium chloride, sodium nitrite, Griess reagent, curcumin, sodium phosphate monobasic and dibasic, tris base and quercetin dihydrate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ascorbic acid, acetic acid glacial, hydrochloric acid, sodium chloride, dimethyl sulfoxide (DMSO), ferrous sulfate ($FeSO_4$), nitro blue tetrazolium, nicotinamide adenine dinucleotide, phenazine methosulphate, ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), ethylenediaminetetraacetic acid disodium dehydrate ($EDTANa_2 \cdot 2H_2O$), Folin-Ciocalteu phenol reagent, and sodium carbonate were purchased from Merck Chemical Co. (Malaysia). Allopurinol, xanthine, xanthine oxidase (bovinemilk) also potassium oxonate were purchased from Sigma-Aldrich Chemicals (St.

Louis, MO, USA). All chemicals used are of analytical grade and were used without further purifications.

3.3 Preparation of Flower Extract

The red flowers of *P. rubra* were cleaned and cut into small pieces for efficient drying process. The red flowers were dried in the shaded at room temperature before grounded into fine powder by using domestic blender and store in the freezer prior the extraction process. The extracts of hexane, chloroform and methanol were prepared by using Soxhlet apparatus.

The ground flowers (22 g) was placed in a thimble-holder, and extracted with 250 ml of solvent in boiling flask at 40 °C for 20 hours. When extraction solvent reach the boiling point, the vapors condensed through the condenser and the solvent drips into the thimble contained grind sample. Extractions were repeated using with different polarity starting with lowest polarity to more polar solvent. The solvent from the filtrate was removed using rotary vacuum evaporator at more than 40 °C to yield hexane, chloroform and methanol extract.

The ground flowers (22 g) was soaked in distilled water with the ratio of 1:10 and at room temperature approximately for 3 days. The extraction was carried out without adding any heat to avoid compound destruction. Following extraction, the water extracts was filtered and the residue was then re-extracted under the same conditions twice. The filtrates were combines and freeze – dried. Water extract of *P. rubra* was extracted accordingly to the method described by Handa, (2008). The dried powder were weighed and kept in sample bottle at -20 ℃ until further used.

3.4 Detection of Phytochemical Compounds

a) Thin Layer Chromatography (TLC)

The separation of phytochemical compounds in red flower of *P. rubra* extract was carried out using Thin Layer Chromatography (TLC) according to method described by Harbone, (1984). The polar stationary phase was more strongly attracted polar molecules.

1 cm was measured from the base of the TLC plate, marked with a pencil and labelled. Capillary tube was used to spot the plates with the extract. Small quantities of the concentrated solutions were collected with capillary tube by dipping it in the solution. They were then used to spot the plates on Silica gel 60 F254–precoated TLC plates, size 20 cm x 20 cm. The plates were placed in a chromatography tank and covered, ensuring that the solvent was just below the spots. Solvent was poured into the TLC chamber to a depth of just less than 0.5 cm and must be covered with watch glass. The following solvent system are used: toluene: hexane (3:7) for hexane extract *P.rubra* (PR–HE), chloroform: methanol (9:1) for chloroform extract *P.rubra* (PR–CE), butanol: acetic acid: water (4:5:1) for methanol extract *P.rubra* (PR–ME) and water extract *P.rubra* (PR–WE). Apparatus was placed on a level surface for the solvent to rise. The plate was removed after about two hours when the solvent had risen close to the top edge, the distance travelled by solvent with a pencil was marked immediately. It was then dried at room temperature and viewed under UV–254 nm and 366 nm.

The distances moved by the solvents and the spots were measured, in centimetres, with a rule. The retardation factors (R_f) of the samples were then determined. The R_f value was the distance of compound moved in chromatography relative to the solvent front, (Figure 3.1) following the formula:

$$R_f \text{ value} = \frac{\text{Distance travelled by chemical compound (B)}}{\text{Distance traveled by solvent front (A)}}$$
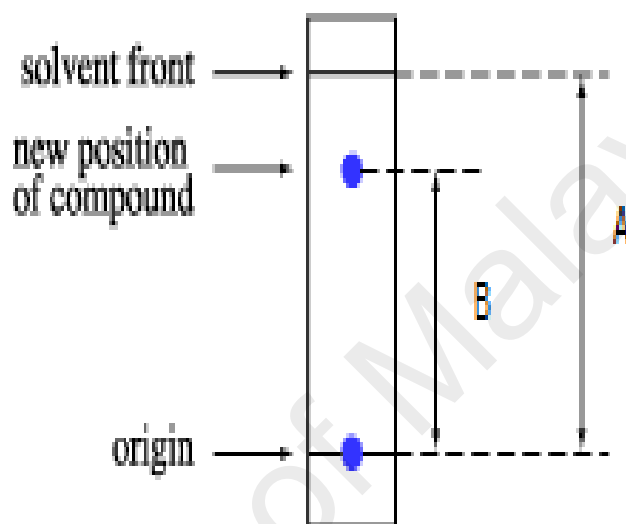
Figure 3.1 Diagram of determination of Retardation factor (R_f) value on TLC plate.

The detection of chemical compound of TLC plates was carried out by observing under visible light and UV light for any colour presence and then TLC plate were dried in fume cupboard prior to spraying with different chemical reagents. Reagents were prepared according to published procedure described by Wagner & Bladt, (2001).

i. Visible light

Under visible light, the coloured spots can be seen clearly on the dried TLC plate. The dried TLC plates are examined initially in visible light for pigments which appear as coloured spots. Available coloured spots on the plate were marked lightly with a pencil and R_f value were measured. The coloured spots were analyzed in order to detect

46

which phytochemical compound and pigments such as chlorophyll, xanthophyll or betalains.

ii. Ultra Violet (UV) light

Those spots which could not be seen under visible light need to be further visualized with ultra violet light. TLC chromatograms were observed under ultra violet light in the dark room by using shortwave UV lamp at 254 nm and long wave UV lamp with 366 nm to detect the fluorescence spots then marked with a pencil and R_f value was measured. TLC plate were observed whether included native florescence which appeared as bright zone on a dark background under long wave at 366 nm or included florescence indicator compounds that can quench the fluorescence and show up as a dark spots or other colours such as purple or blue on a green background under short wave at 254 nm (Harbone, 1984).

iii. Dragendroff's Reagent

The mixture of 0.8 g of bismuth nitrate ($BiONO_3$) in 10 mL of glacial acetic acid (HAc) was diluted with distilled water in order to obtained solution A. Solution B was prepared by dissolving 20 g Potassium iodide (KI) powder to 50 mL distilled water in brown volumetric flask. 100 mL dragendroff reagent was prepared according to Jia and Tia, (2009) by mixtures of 5 mL solution A and 5 mL solution B in 100 mL brown volumetric flask, and mixtures was diluted until 100 mL solution. Dragendroff reagent was stored in covered brown bottle.

TLC plates which had been removed from TLC chamber were placed in the fume cupboard in order to allow the solvent to evaporatate before they were sprayed with dragendroff reagent. The reagent reacted to alkaloids and choline produced orange spots.Orange spots were marked with a pencil and R_f value were measured.

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iv. Vanilin–Sulphuric acid reagent

Fresh vanillin–sulphuric acid (H_2SO_4) reagent was prepared by dissolving 1 g of vanillin in 100 mL of ethanol and water (40:10) .TLC plates which had been removed from TLC chamber were placed in the fume cupboard in order to allow the solvent to evaporatate before they were sprayed with vanillin–H_2SO_4 reagent. Then, the plates were heated on hot plate at 100 °C. The formation of purple spot indicate presence of terpenoid compounds while red and pink spots indicates presence of phenolic compounds

v. Anisaldehyde – Sulphuric Acid reagent

A mixture of 0.5 mL of anisaldehyde, 80 mL methanol, 30 mL of glacial acetic acid (HAc) and 5 mL of H_2SO_4. TLC plates which had been removed from TLC chamber were placed in the fume cupboard in order to allow the solvent to evaporatate before they were sprayed with anisaldehyde–H_2SO_4 and heated at 100 °C. The formation of red spot indicates the presence of flavonoids, purple spot indicates the presence of terpenoids while blue and black spot indicate the presence of saponins.

b) Liquid Chromatography Mass Spectrometry (LCMS)

The PR–ME and PR–WE extracts from red flower of *P.rubra* was analyzed with Liquid Chromatography Mass Spectrophotometry (LCMS) to determine the chemical compounds present. Extracts was screened with AB Sciex 3200QTrap LCMS/MS and fully scan with MS/MS data collection with negative ionization mode. Phenomenex Aqua C18 (50 mm x 2.0 mm x 5 µM) was used a column in a rapid screening at 15 min run time. Water with 0.1 % formic acid (HCO_2H) and 5 mM ammonium formate (NH_4HCO_2) was used as buffer A and a mixture of acetonitrile (CH_3CN) with 0.1 %

HCO$_2$H and 5 mM ammonium formatted as buffer B. Sample were run with gradient mode; 10 % A to 90 % B from 0.01 min to 8.0 min and were held for 3 min and back to 10 % A in 0.1 min and re−equilibrated for 4 min. Pre- run equilibration time was 1.0 min. 1 mL of concentrated sample of PR−ME and PR−WE extracts was diluted 5 times with methanol and filtered with 0.2 µM nylon filter prior to being analyzed. Injection volume for both samples were 20 µL.

3.5 Determination of Total Phenolic Content

Total phenolics were determined using Folin−Ciocalteu (FC) reagent according to the method described by Muller et al (2010) with slight modification.

3.5.1 Preparation of Folin−Ciocalteu (FC) Reagent

FC reagent was prepared by mixing 20 mL of the folin−ciocalteu reagent with 200 mL distilled water.

3.5.2 Preparation of 7.5 % Sodium Carbonate (Na$_2$CO$_3$)

Na$_2$CO$_3$ was prepared by dissolving 7.5 g of Na$_2$CO$_3$ stock in 100 mLdistilled water.

3.5.3 Preparation of Gallic Acid 1mg/mL Standard solution

Gallic acid was prepared by dissolving 0.01 g of gallic acid in 10 mL distilled water used as positive control.

3.5.4 Total Phenolic Assay

Briefly, twenty microliters of red flower from *P. rubra* extracts was mixed with 100 μL of FC reagent (diluted 10-fold with distilled water) in a 96–well microplate, incubated for 5 min, and 75 μL of Na_2CO_3 solution (7.5 %) was added. After 2 hours of incubation period in darkness at room temperature, the absorbance was measured at 740 nm using microplate reader (Tecan Sunrise, Austria). Gallic acid (1 mg/1mL) was used as standard for calibration and construction of a linear regression line and DMSO 3 % was used as a blank. The total phenolic content of the extract was calculated as mg gallic acid equivalent (GAE) mg /g of dry weight extract and were done in triplicate.

3.6 Determination of Total Flavonoid Content

The total flavonoid content was determined according to the method described by Zhishen *et al.* (1999) with slight modification.

3.6.1 Preparation of 5 % Sodium nitrite (NaNO₂) solution

5 % of $NaNO_2$ was prepared by dissolving 5 g $NaNO_2$ in 100 mL distilled water.

3.6.2 Preparation of 10 % Aluminium Chloride (AlCl₃)

10 % $AlCl_3$ was prepared by dissolving 10 g $AlCl_3$ in 100 mL distilled water.

3.6.3 Preparation of 1M Sodium hydroxide (NaOH) solution

1M NaOH solution was prepared by dissolving 1.599 g NaOH in 60 mL distilled water.

3.6.4 Total Flavonoid Assay

Briefly, fifty microliters of extracts were added with 70 μL of distilled water and 15 μL of 5 % NaNO$_2$ solution in a 96–well microplate. The solutions were well mixed and incubated for 5 min at room temperature. Then, 15 μL of 10 % AlCl$_3$ solution was added into the mixture. After 6 min of incubation, 100 μL of 1M NaOH solution was added, and the absorbance was measured at 510 nm with a microplate reader (Tecan Sunrise, Austria). Methanol was used as blank. The final absorbance of each extracts was compared with a standard curve plotted of quercetin. The total flavonoid content of the extracts was expressed in mg quercetin equivalent (QAE) mg /g of dry weight extract.

3.7 Determination of Antioxidant Activity

3.7.1 2, 2–diphenyl–1–picrylhydrazyl (DPPH) Scavenging Activity Assay

Quantitative measurements of radical scavenging assay were carried out according to the method as described by Marghitas *et al.* (2009) with a slight modification.

a. Preparation of 50 μM DPPH solution in methanol

0.00197 g of DPPH was dissolved in 100 mL methanol. The solution was kept in flask wrapped with aluminium foil. This reagent is stable when stored in a refrigerator at 4 °C for several days.

b. DPPH Scavenging Assay

The scavenging activity of red flowers from *P. rubra* extracts and ascorbic acid were tested at various concentrations range of 15.63, 31.25, 62.5, 125 and 250 μg/mL. Reaction mixtures containing DPPH and extracts or ascorbic acid were prepared according to Table 3.1.

Table 3.1 Preparation of Reaction Mixtures of Extracts or Ascorbic acid and DPPH reagent for DPPH Scavenging Assay

Concentrations of ascorbic acid or extracts (μg/mL)	Volume of ascorbic acid or extracts (μL)	Volume of DPPH reagent (μL)
250	40	200
125	40.	200
62.5	40	200
31.25	40	200
15.63	40	200

Briefly, 40 μL of extracts of each concentrations (15.63 to 250 μg/mL) were mixed with 200 μL of 50 μM DPPH solution in methanol. The mixture was immediately shaken and incubated for 15 min in the dark at room temperature. The absorbance reading was measured at 517 nm using ELISA microplate reader (Tecan Sunrise, Austria). Ascorbic acid (15.63–250 μg/mL) served as a standard positive control and the negative control was methanol.

The percentage of scavenging activity of the *P. rubra* flowers extracts was calculated according to the following equation:

DPPH scavenging activity (%) =

$$\frac{\text{Absorbance of control} - \text{Absorbance of sample / standard}}{\text{Absorbance of control}} \times 100\%$$

The concentration of extracts required to scavenge 50 % of DPPH radical was estimated from the graph plotted against the percentage inhibition and compared with the standard. All the tests were performed in triplicate, and the results were expressed as μ/mL.

3.7.2 Ferric Reducing Anti-oxidant Power (FRAP)

The FRAP assay was carried out as described according to Benzie & Strain (1996) with slightly modification in 96–well microplates.

a. Preparation of Acetate Buffer 0.3M

16 mL of glacial acetic acid was added to 3.1 g sodium acetate trihydrate. Then, solution was made up to 1 L using distilled water. The pH of the solution was adjusted using pH meter.

b. Preparation of 2,4,6–Tripyridyl–s–Triazine (TPTZ) solution

0.031 g of TPTZ was added to 10 mL of 40 mM HCl and dissolved at 50 °C in water bath. TPTZ solution was freshly prepared each time the assay was performed.

c. Preparation of Ferric chloride solution (FeCl₃)

0.054 g of FeCl$_3$ was dissolved in 10 mL of distilled water. FeCl$_3$ solution was freshly prepared each time the assay was performed.

d. Ferric Reducing Anti-oxidant Power (FRAP) assay

Twenty microliters of red flowers of *P. rubra* extracts in ethanol were mixed with 200 μL of daily prepared FRAP reagent, which contained 5 mL 10 mM TPTZ in 40 mM HCl, 5 mL of 20 mM FeCl$_3$, and 50 mL of 0.3M acetate buffer (pH3.6) in 96–well microplate. After 8 min of incubation time, the formation of the TPTZ–Fe^{2+} complex in the presence of antioxidant compounds in the extract was measured at 595 nm with a

microplate reader (Tecan Sunrise, Austria). Methanol was used as a blank. Ferrous sulfate (FeSO$_4$) solution (0.2 mM to 1 mM) was used for standard calibration curve. The FRAP value was evaluated according to the linear regression between standard solutions and absorbance at 595 nm and the results were estimated as mmol Fe$^{2+/}$ g of dry extract from triplicate tests.

3.7.3   Metal Chelating Activity Assay

The ferrous ion chelating activity of the red flowers from *P. rubra* extracts was determined according to the procedure by Srivastava *et al.* (2012) by measuring the formation of the Fe^{2+}−Ferrozine complex. The assay performed to measure chelating ability of ferrous ion was based on the chelation of ion Fe^{2+} with ferrozine to form ferrous-ferrozine complex which can be detected at 562 nm.

a.   Preparation of 5 mM of Ferrozine (FZ)

A stock of 0.0246 g of FZ was dissolved in 10 mL deionized water. The stock solution was kept in centrifuge tube and wrapped with aluminium foil.

b.   Preparation of 2 mM Ferum Chloride (FeCl$_2$)

A stock of 0.0025 g of FeCl$_2$ was dissolved in 10ml deinonized water. The stock solution was kept in centrifuge tube and wrapped with aluminium foil.

c. Metal Chelating Assay

EDTA was used as positive reference standard in metal chelating assay. The metal chelating activity of red flowers *P. rubra* extracts and EDTA were tested at

various concentrations of 15.63, 31.25, 62.5, 125 and 250 μg/mL. Reaction mixtures containing ferrozine, ferric chloride in Ethylenediaminetetraacetic acid (EDTA) or extracts were prepared according to Table 3.2.

Table 3.2 Preparation of Reaction Mixtures of Extracts or EDTA Containig Ferrozine, Ferric Chloride and Distilled Water.

Concentration of EDTA or extract (μg/mL)	Volume of Ferrozine (FZ) (μL)	Volume of Ferric Chloride (FeCl$_3$) (μL)	Volume of EDTA or extracts	Volume of distilled water (μL)
250	20	10	100	120
125	20	10	100	120
62.5	20	10	100	120
31.25	20	10	100	120
15.63	20	10	100	120

Briefly, 100 μL of extracts from each concentrations (15.63–250 μg/mL) were mixed with 120 μL distilled water and 10 μL FeCl$_2$ (2 mM) in a 96–well microplate. FZ (5 mM, 20 μL) was added to the mixture to initiate the reaction. The reaction mixture was incubated at room temperature for 20 min and absorbance at 562 nm. EDTA was served as positive control (15.63–250 μg/mL) while 0.05 % DMSO was used as a negative control; blank was without ferrozine (20 μL of distilled water instead of FZ). The percent inhibition of Fe $^{2+}$–FZ complex formation was calculated. The concentration of extracts required to chelate 50 % of the Fe^{2+} ion (IC$_{50}$) was calculated from the graph against the percentage of inhibition.

The percent inhibition of Fe^{2+-}–FZ complex formation was calculated according to the following formula:

Ferrous ion chelating activity =

$$\frac{\text{Absorbance control} - (\text{Absorbance sample or standard})}{\text{Absorbance control}} \times 100$$

3.7.4 Hydrogen peroxide Scavenging Activity Assay

The scavenging capacity for hydrogen peroxide was measured according to the method as described by Sroka & Cisowski, (2003) with a slight modification.

a. Preparation of 50mM Phosphate buffer

Phosphate buffer was prepared according to phosphate buffered saline system, are comprising the following reagents: potassium phosphate (monobasic) (KH_2PO_4), potassium phosphate (dibasic) (K_2HPO_4) and sodium chloride (NaCl)

Solution A : 1.36 g KH_2PO_4 (monobasic) was dissolved in 10 mL distilled water.

Solution B : 8.70 g K_2HPO_4 (dibasic) was dissolved in 50 mL distilled water

Solution C : 2.93 g NaCl was dissolved in 50 mL distilled water.

6.3 mL of solution A, 43.7 mL of solution B and 30 mL of solution C were mixed and diluted with one liter of distilled water, then, the pH of buffer was adjusted to 7.4.

b. Preparation of 2 mM Hydrogen peroxide (H_2O_2) solution

100 mL of 2 mM H_2O_2 was prepared with the mixtures of 20 mL of hydrogen peroxide solution and 80 mL phosphate buffer.

c. Hydrogen peroxide Scavenging Assay

The scavenging activity of both red flowers from *P. rubra* extracts and ascorbic acid were tested at various concentrations of 15.63, 31.25, 62.5, 125 and 250 μg/mL. Reaction mixtures containing H_2O_2 solutions in phosphate buffer (50 mM) and extracts or ascorbic acid were prepared according to Table 3.3.

Table 3.3 Preparation of Reaction Mixtures of Extracts or ascorbic acid and hydrogen peroxide solution

Concentration of ascorbic acid or extracts (μg/mL)	Volume of ascorbic acid or extracts (mL)	Volume of Hydrogen Peroxide (H_2O_2) solution (mL)
250	0.1	0.6
125	0.1	0.6
62.5	0.1	0.6
31.25	0.1	0.6
15.63	0.1	0.6

A solution of H_2O_2 (2 mM) was prepared in 50 mM phosphate buffer (pH 7.4). H_2O_2 concentration was determined spectrophotometrically at 230 nm absorption using the molar extinction coefficient for H_2O_2 of 81 $mol^{-1}cm^{-1}$. 0.1 mL of each concentrations of extracts and ascorbic acid (16.63−250 μg/mL in respective solvents), was transferred into the test tubes and their volumes were made up to 0.4 mL with 50 mM phosphate buffer (pH 7.4) or solvent (methanol). After addition of 0.6 mL H_2O_2 solution, tubes were vortexed and absorbance was measured at 230 nm was determined after 10 min, against a blank. 50 mM phosphate buffer without H_2O_2 was used as blank.

Hydrogen peroxide scavenging ability (in triplicate) was calculated by the formula:

Hydrogen peroxide scavenging activity =

$$\frac{\text{Absorbance control} - (\text{Absorbance sample/ standard})}{\text{Absorbance Control}} \times 100$$

3.7.5 Nitric Oxide Scavenging Activity Assay

The nitric oxide scavenging activity of the red flowers from *P. rubra* extracts was determined according to the procedure by Srivastava *et al.* (2012) by measuring the formation of the nitrite ions in the reaction mixture that can be detected by Griess reagent which can be detected at 546 nm.

a. Preparation of 20mM Phosphate Buffered Saline (pH 7.4)

0.1 M Phosphate buffer was prepared according to phosphate buffered saline system, are comprising the following reagents: potassium phosphate (monobasic) (KH_2PO_4), potassium phosphate (dibasic) (K_2HPO_4) and Sodium Chloride (NaCl).

Solution A   : 27.6 g of KH_2PO_4 (monobasic) was dissolved in 19 mL distilled water.

Solution B   : 28.4 g K_2HPO_4 (dibasic) was dissolved in 81 mL distilled water

Solution C   : 4.68 g NaCl was dissolved in 80 mL distilled water

Solution A, Solution B and Solution C were mixed and diluted with 100 mL of distilled water, then, the pH of buffer was adjusted to 7.4.

20 mM phosphate buffer was prepared by diluted 10 mL 0.1 M phosphate buffer with 40 mL distilled water. The pH of buffer was adjusted to 7.4.

b. Preparation of 10 mM of sodium nitroferricyanide ($Na_2[Fe(CN)5NO] \cdot 2H_2O$)

A stock of 0.149 g sodium nitroferricyanide was dissolved in 50 mL phosphate buffer.

c. Preparation of Griess reagent

0.2 % naphthylethylenediamine dihydrochloride and 2 % sulphanilamide in 5 % phosphoric acid were prepared separately in an amber colored bottle label it and store it in 4 °C. The equal volume of both solutions were mixed before assay estimation.

d. Nitric Oxide Radical Scavenging Assay

Curcumin was used as positive reference standard in metal chelating assay. The nitric oxide radical scavenging activity of each *P. rubra* flowers extracts and curcumin were tested at various concentrations of 15.63, 31.25, 62.5, 125 and 250 μg/mL. Reaction mixtures containing sodium nitroferricyanide (Na$_2$[Fe(CN)5NO] · 2H$_2$O) and Griess reagent with curcumin or extracts were prepared according to Table 3.4.

Table 3.4: Preparation of Reaction Mixtures of Extracts or Curcumin Containing Sodium ferrocyanide and Griess Reagent

Concentration of Curcumin or extract (µg/mL)	Volume of Sodium ferricyanide (Na$_2$[Fe(CN)5NO] · 2H$_2$O (µL)	Volume of Griess reagent (µL)	Volume of Curcumin or extracts (µL)
250	50	125	50
125	50	125	50
62.5	50	125	50
31.25	50	125	50
15.63	50	125	50

Briefly, fifty microliters of extracts at each concentrations (15.63−250 μg/mL) and an equal amount of sodium nitroferricyanide (Na$_2$[Fe(CN)5NO]·2H$_2$O) (10 mM) in phosphate-buffered saline (20 mM, pH 7.4) were mixed well in a 96−well microplate. The mixture was incubated at room temperature for 150 min and 125 μL of Griess reagent was added. After 10 min, the absorbance was measured at 546 nm with a microplate reader (Tecan Sunrise, Austria). Curcumin (15.63−250 μg/mL) and ethanol

were used as a standard and control. The reaction mixture without Griess reagent was served as blank.

The percent inhibition of nitric oxide was calculated using the formula:

$$\frac{\text{Absorbance control} - (\text{Absorbance sample or standard})}{\text{Absorbance control}} \times 100\%$$

The concentration of extracts needed to scavenge 50 % of the nitric oxide (IC_{50}) was estimated from the graph against the percentage of inhibition. All the tests were performed in triplicate, and the results were expressed as μg/mL.

3.7.6 Superoxide Scavenging Activity Assay

The superoxide scavenging activity was determined by PMS – NADH with slightly modifications as described by Shukla *et al.* (2012). Superoxide radicals are generated in phenazine methosulphate-nicotinamide adenine (PMS–NADH) systems by the oxidation of NADH and assayed by the reduction of NBT which can be measured at 570 nm.

a.   Preparation of 0.1M Tris-HCl Buffer

The stock solution buffer was prepared 121.11 g Tris base was dissolved in 800 mL distilled water. The desired pH was adjusted with concentrated 1M HCl. The volume was adjusted to 1 L with distilled water. 0.1M was prepared by dilution of 5 mL of 1M Tris–HCl buffer with 45 mL of distilled water. The desired pH (pH 8) was adjusted with concentrated 1M HCl.

b.   Preparation of 0.2 mM Nitro blue tetrazolium (NBT) solution

The stock of 0.0016 g NBT was dissolved in 10 mL distilled water.

c.   Preparation of 0.5 mM Nicotinamide adenine dinucleotide (NADH) solution

The stock of 0.0038 g NADH was dissolved in 10 mL Tris–HCl buffer.

d.   Preparation of 25 µM phenazine methosulphate (PMS) solution

The stock of 0.0007 g PMS was dissolved in 10 mL Tris–HCl buffer.

e.   Superoxide Radical Scavenging Assay

Quercetin and ascorbic acid were used as positive reference standard in superoxide radical scavenging activity assay. The red flowers of *P. rubra* extracts as well as standards, quercetin and ascorbic acid were tested at various concentrations of 15.63, 31.25, 62.5, 125 and 250 *μ*g/mL. Reaction mixtures containing with PMS, NADH and NBT reaction system with quercetin, ascorbic acid or extracts were prepared according to Table 3.5.

Table 3.5: Preparation of Reaction Mixtures for Superoxide Radical Scavenging Assay

Concentration of Quercetin or Ascorbic acid or extracts (μg/mL)	Volume of Quercetin or Ascorbic acid or extracts (μL)	Volume of Nitro blue tetrazolium (NBT) (μL)	Volume of Nicotinamide adenine dinucleotide (NADH) (μL)	Volume of Phenazine methosulphate (PMS) (μL)
250	100	50	50	50
125	100	50	50	50
62.5	100	50	50	50
31.25	100	50	50	50
15.63	100	50	50	50

Briefly, fifty microlitre of NBT solution (0.2 mM in distilled water) with 50 µL of NADH solution (0.5 mmol/L in 0.1 M Tris–HCl, pH 8.0) and 100 µL of extract at each concentrations (15.63–250 µg/mL) were mixed and treated with 50 µL of PMS solution (25 µM PMS in distilled water). The reaction mixture was incubated at room temperature for 10 min, and the absorbance at 570 nm was measured. Quercetin and ascorbic acid were used as positive control.

The percentage of scavenging was calculated by the formula:

$$\frac{A_1 - A_0}{A_1} \times 100\%$$

The A_1 was the absorbance of control (PMS–NADH–NBT reaction system without sample) at 517. A_0 was the absorbance at 570 nm with sample at different concentrations with PMS–NADH–NBT reaction system.

The concentration of extracts needed to scavenge 50 % of the nitric oxide (IC_{50}) was estimated from the graph against the percentage of inhibition. All the tests were performed in triplicate, and the results were expressed as μg/mL.

3.8   Xanthine Oxidase Inhibitory Activity

3.8.1   *In vitro* Model

The XO inhibitory assay of the extracts from *P. rubra* red flowers was determined according to the procedure by Owen & Johns, (1999) and all these extracts from *P.rubra* flower were asayed spectrophotometrically under aerobic conditions.

a.   Preparation of 50 mM Phosphate buffer

Phosphate buffer was prepared according to phosphate buffered saline system, are comprising the following reagents: potassium phosphate (monobasic) (KH_2PO_4), potassium phosphate (dibasic) (K_2HPO_4) and sodium Chloride (NaCl)

Solution A   : 1.36 g KH_2PO_4 (monobasic) was dissolved in 10 mL distilled water.

Solution B   : 8,70 g K_2HPO_4 (dibasic) was dissolved in 50 mL distilled water

Solution C   : 2.93 g NaCl was dissolved in 50 mL distilled water.

6.3 mL of solution A, 43.7 mL of solution B and 30 mL of solution C were mixed and diluted with one liter of distilled water, then, the pH of buffer was adjusted to 7.5.

b.   Preparation of Xanthine Oxidase (XO) enzyme solution

5 mL Phosphate buffer was mixed w ith 1 unit/mL XO to produced 0.1 unit/mL stock of XO enzyme solution.

1 mL from stock enzyme solution was mixed with 9 mL Phosphate buffer to produced 0.01 unit/mL of XO enzyme solution.

c.   Preparation of Xanthine substrate (150mM) solution

150 mM of xanthine was prepared by dissolving 0.456 g of xanthine in 20 mL phosphate buffer.

d.   Preparation of 1N of Hydrochloric acid (HCl)

1N of HCl was prepared by mixed 82.898 mL HCl with 917.102 mL distilled water.

e.   Xanthine Oxidase Inhibition Activity *in vitro* Assay

Briefly, 1 ml of extracts at each concentration (62.5 to 500 µg/mL) was added to 2.9 mL of phosphate buffer, and 0.1 mL of enzyme solution (0.01 units/ mL in phosphate buffer, pH 7.5) which was prepared immediately before use. After pre‒incubation at 25°C for 15 min, the reaction was initiated by the addition of 2 mL of 150 mM xanthine solution in the same buffer which acts as a substrate and this reaction mixture were incubated at 25 °C for 30 min. The reaction was stopped by addition of 1 mL of 1N of HCl and the absorbance was measured at 290 nm by using UV spectrophotometer. Blank was prepared in the same way, but the enzyme solution added to the assay mixture after adding 1N HCl. The assay was done in triplicate. One unit of

XO is defined as the amount of enzyme required to produce 1 mmol of uric acid per min at 25˚C.

XOI activity was evaluated as the percentage of inhibition of XO in this assay system calculated as:

Percentage of XO inhibitory activity =

$$\frac{(A - B)}{(C - D)} \times 100\%$$

Where A is the activity of the enzyme without test extract, B the control of A without test extract and enzyme, C and D are the activities of the test extract with and without XO, respectively.

The extracts were dissolved initially in DMSO followed by dilution with the buffer and the final concentration of DMSO was less than 0.5 %. Allopurinol, a known inhibitor of XO, was used as a positive control.

3.8.2 *In Vivo* Model

a. Rat For *In Vivo* Study

Male Sprague–Dawley (SD) rats (200 ± 30 g) were purchased from the University of Malaya Animal Experimental Unit. Rats were allowed to adapt to their environment before being used for experiments for at least 1 week. They were maintained in a room controlled at 22–24 ℃ with a relative humidity of 60 ± 5 % and a 12 hours light/ dark cycle (6:00 a.m– 6:00 p.m.). They were given standard chow and water ad libitum for the duration of the experiment. All experimental protocols described in this study were approved by Ethics Committee on Animal Experiment of University of Malaya Animal Care and Use Committee. (Ethic No ISB/12/09/2014/SSPMI (R))

b. Acute Toxicity Study

Acute toxicity study of PR – ME extract of red flower *P.rubra* was performed in healthy adults Sprague Dawley (SD) rats according to OECD guidelines 423 (Organization of Economic Cooperation and Development in 2001. A 6 of healthy adult male and female of SD rats were kept overnight fasted (16 hours) but had been allowed to free access to water prior to extract administration using an intragastric tube. After the administration of the extract, food but not water is withheld for further 3 to 4 hours. The group of acute toxicity study of the present study were divided into two groups. Group 1 was treated orally with extract 2 g/kg body weight. Group 2 was treated orally with extract 4 g/kg body weight. The rats were observed at least once during the first 24 hours which the special attention to first 4 hours and also daily thereafter for a period of 14 days. Observations of first 4 hours should include changes in skin, fur, eyes and mucous membranes, and also respiratory, autonomic including defecation and urination,

the central nervous systems including spontaneous reaction, reactivity, touch response, pain response, and the behavior pattern including alertness, restlessness and irritability. Mortality, if any, is determined over a period of 2 weeks. The LD50 of the extract as per OECD guidelines falls under class four values with no sign of acute toxicity at 4 g/kg. The biological evaluation is carried out at 1/10th of maximum tolerated dose i.e. 400 mg/kg body weight if no toxic signs observed in rats.

c.   *In Vivo* **Study of Hyperuricemic Rats**

The hypouricemic effect of PR – ME extract from red flower *P. rubra* was examined by the methods reported previously by Liu *et al.* (2014) with slight modification. The hyperuricemic rat model was prepared by intraperitoneal (i.p) administration of potassium oxonate (PO) (280 mg/kg single dose) 1 hour before the final drug administration according to the method described by Carroll *et al.* (1971). PO for administration was suspended in 0.9 % NaCl solution and was given at day 1, 3, 5 and 7. Allopurinol solution, extract solutions and PO suspension were prepared according to the body weight measured immediately prior to drug administration. Reaction of allopurinol and *P. rubra* flower doses as hyporuricemic activity were determined based on conversion from human clinical practice. *P. rubra* extract and allopurinol were solubilized in 0.5 % carboxymethylcellulose (CMC) prior to drug administration. All drug and extract solution were given orally once daily at 11.00–12.00 hour within seven consecutive days.

Briefly, the eight groups of SD rats are assigned to this experiment (n=6 per group). Generally group 1, 2 and 3 are normal control groups which are not induced by PO. Then, hypouricemic and xanthine oxidase (XO) activity of methanol extract of *P. rubra* red flower extract was determined. Group 1 served as a baseline which do not

received any solvent vehicle and treatment within seven days of experiment. Group 2 served as a vehicle control group of normal rat which only orally administered with 0.5 % CMC solution for seven consecutive days. In group 3, the rats only receive test extract of *P.rubra* in high dose (400 mg/kg body weight) whereas in group 4 also only received test extract but in low dose (100 mg/kg body weight). Group 5 is hyperuricemia group served as gout control which intraperitoneally administered PO in a dose of 280 mg/kg without received any treatment. Group 6 and 7 received the oral gavage of anti- gout treatment with extract solution which high dose (400 mg/kg body weight) and low dose (200 mg/kg body weight), respectively. Group 8 is a standard drug control which received allopurinol (10 mg/kg body weight). The treatment for group 6, 7 and 8 were given after 1 hour induced with PO. At the seventh day, two hours after PO−induced action, the rats were anaesthetized with ketamine and xylazine (100 and 20 mg/kg, respectively), via intraperitoneal injection and whole blood samples will be collected by cardiac puncture 1 hour later after final drug administration. The blood will be allowed to clot for 1 hour at ambient temperature and then centrifuge at 3500 rpm for 5 min to obtain the serum. The serum was stored at -40 °C until assayed.

Table 3.6. Summary of Classification Group of Rats and their Treatment in Seven Days of *In Vivo* Study

Number of groups	Classification	Treatment
1	Baseline (N)	No solvent vehicle, standard allopurinol and *P.rubra* extracts administration
		Non-induced with PO
2	Vehicle Control (0.5 % CMC)	0.5 % of CMC administration
		Non-induced with PO
3	High Dose Control Group of *P.rubra* extracts (HC)	400 mg/kg body weight of *P.rubra* extracts administration
		Non-induced with PO

4	Low Dose Control Group of *P.rubra* extracts (LC)	200 mg/kg body weight of *P.rubra* extracts
		Non- induced with PO
5	Hyperuricemic Gout Control Group (HGC)	Intraperitoneally administration of PO in dose of 280 mg/kg at day 1, 3, 5 and 7
		No administration of *P.rubra* extracts and standard allopurinol
6	High Dose Treatment Group of *P.rubra* Extracts (HPRT)	Intraperitoneally administration of PO in dose of 280 mg/kg at day 1, 3, 5 and 7
		400 mg/kg body weight of *P.rubra* extracts administration
7	Low Dose Treatment Group of *P.rubra* extracts (LPRT)	Intraperitoneally administration of PO in dose of 280 mg/kg at day 1, 3, 5 and 7
		200 mg/kg body weight of *P.rubra* extracts administration
8	Standard Drug Group Allopurinol (AP)	Intraperitoneally administration of PO in dose of 280 mg/kg at day 1, 3, 5 and 7
		10 mg/kg body weight of standard drug allopurinol administration

3.9 Body Weight Measurement of Rats

Fasting body weight (BW) of rats were monitored for every 2 days until final administration of drugs which held on day 0, day 1, day 3, day 5 and day 7. The *in vivo* studies were carried out in 16 hours fasted rats.

3.10 Determination of Uric Acid Assay

Serum uric acid concentration was determined by enzymatic colorimetric method, using a standard diagnostic kit purchased from Sigma – Aldrich (MAK077) according to manufacturer's instructions.

3.11 Determination of Xanthine Oxidase Assay in *In Vivo* Model

Rats livers were excised immediately after blood collection, washed in 0.9 % cold saline and rapidly stored at - 80 °C until further experiment. Briefly, livers were homogenized in 4 mL of 80 mM sodium phosphate buffer (pH 7.4) and, then, the homogenate was centrifuged at 3500 g for 10 min at 4 °C. Lipid layer was carefully removed, and supernatant was further centrifuged at 10,000 g for 60 min at 4 °C. The final supernatant was used for xanthine oxidase enzyme activity assays. Xanthine oxidase activity was determined spectrophotometrically using standard kit purchased from Sigma- Aldrich (MAK078).

3.12   Statistical Analysis

Results were expressed as the mean ± standard error (SEM) for the three independent experiments. Differences between extracts were analyzed by one way ANOVA followed by Duncan and Dunnett's post hoc multiple comparison test at the 5 % level ($p < 0.05$). The statistical program (SPSS 22.0 version, Chicago, IL, USA) was performed in the entire test.

CHAPTER 4

RESULTS

4.1 Extraction Yields of *Plumeria rubra* flowers extracts

Dry powdered of red *P. rubra* flowers (22 g) was extracted with hexane, chloroform and methanol using a Soxhlet apparatus for 20 hours and the filtrate were evaporated under reduced pressure using rotary evaporator to remove the solvent. From the extraction process, a variety of extraction yield of *P. rubra* flower extract from different solvents were obtained. The hexane (PR−HE) extract was oily yellowish green with 3.27 % of yield, chloroform (PR−CE) extract is a greenish brown with 3.03 % yield and methanol (PR−ME) extract is dark purple powder with 10.76 % yield.

The water (PR − WE) extract of *P. rubra* flowers was prepared by incubating the powder in water bath at 40 °C for 8 hours. The yield obtained after lyophilization was a dark red powder with 7.35 % yield. The extraction yields, in descending order, were: PR−ME > PR−WE > PR−CE > PR−HE. The yields of each extracts were summarized and presented in Table 4.1.

Table 4.1 Yields of *P. rubra* flower extracts

Extract	Yield (%)
PR-HE	3.27
PR-CE	3.03
PR-ME	10.76
PR-WE	7.35

4.2 Detection of Phytochemical compounds from *P.rubra* Flowers

a. Thin Layer Chromatography

The extracts of red *P. rubra* flowers was subjected to TLC method by using TLC aluminium silica gel 60 F_{254} sheets. The presence of chemical compound was detected by spraying sheets with specific visualization reagents such as dragendroff reagents, folin, vanilin- sulphuric acid and anisaldehyde-sulphuric acid. 30 % ethyl acetate in toluene (ethyl acetate: toluene / 3:7) was used as solvent system in TLC to separated one labelled compound in PR–HE extract; PR–HE1; three labelled compounds in PR–CE extract; PR–CE1, PR–CE2 and PR–CE3, seven labelled compounds in PR–ME extract; PR–ME1, PR–ME2, PR–ME3, PR–ME4, PR–ME5, PR–ME6 and PR–ME7;two labeled compound from PR–WE extract; PR–WE1 and PR–WE2.

According to table 4.2–4.5, the appearance of spots in the visible lights showed the presence of the pigments carotenoids which gave the colour of yellow, green and red as well as the flavonoids which gave the colour of red to purple. While the appearance of yellow to green spots under UV light showed the presence of the phytochemical with compound with native florescence which including polycyclic aromatic hydrocarbons, phenols and quinine.

Table 4.2 TLC Profile of PR–HE extract of *P. rubra* Red flowers using Solvent System (ethyl acetate: toluene / 3:7)

Label Compounds	Rf value x 100	Colour under visible light	Color under UV light	Reagent Dragendorff's	Reagent Folin	Reagent Vanilin– H_2SO_4	Reagent Anisaldehyde– H_2SO_4	Comment
				Observation				
PR–HE1	69	-	Yellow (++)	-	Blue (+)	Blue (+)	Blue (+)	Phenol Essential oil

Indication for Intensity of colour:

+++ = Strong

++ = Moderate

+ = Weak

- = No colour observed

Table 4.3 TLC Profile of PR–CE extract of *P. rubra* Red flowers using Solvent System (chloroform: methanol /9:1)

Label Compounds	R_f Value x 100	Visible light	UV light	Reagent Dragendorff's	Reagent Folin	Reagent Vanilin–H_2SO_4	Reagent Anisaldehyde–H_2SO_4	Comment
					Observation			
PR–CE1	64	Green (++)	Yellow (++)	-	Blue (+++)	Blue (++)	Yellow (+)	Phenol
PR-CE2	58	Green (++)	-	-	Blue (++)	Red (+)	-	Phenol
PR-CE3	54	Yellow (+)	-	-	Blue (+)	Red(+)	Red (+)	Phenol, Flavonoid

Indication for Intensity of colour:

+++ = Strong

++ = Moderate

+ = Weak

- = No colour observed

Table 4.4 TLC Profile of PR–ME extract of *P. rubra* Red flowers using Solvent System (butanol: acetic acid: water / 4:5:1)

Label Compounds	Rf Value x 100	Observation						Comment
		Visible light	UV light	Reagent Dragendorff's	Reagent Folin	Reagent Vanilin–H_2SO_4	Reagent Anisaldehyde –H_2SO_4	
PR–ME1	62	Red (++)	Green (++)	-	Blue (+++)	Blue (++)	-	Phenol
PR–ME2	58.1	-	Yellow (+)	-	Blue (+)	Green (+)	-	Phenol
PR–ME3	50.9	Yellow (++)	Green (+)	-	Blue (++)	Green (+++)	Red (+++)	Phenol, Flavonoid
PR–ME4	42.7	Red (+++)	Yellow (+++)	-	Blue (++)	Green (++)	Yellow (+)	Phenol
PR–ME5	32	Purple (+)	Green (+)	-	Blue (+++)	Green (++)	Red (+)	Phenol Flavonoid
PR–ME6	24.5	Green (+)	-	-	Blue (++)	Purple (+)	-	Phenol Terpenoid
PR–ME7	16.4	Yellow (+)	-	-	Blue (+)	Blue (+)	-	Phenol

Indication for Intensity of colour:

+++ = Strong

++ = Moderate

+ = Weak

- = No colour observed

Table 4.5 TLC Profile of PR–WE extract of *P. rubra* Red flowers using Solvent System (butanol : acetic acid:water / 3:1:1)

Label Compounds	Rf Value x 100	Visible light	UV light	Reagent Dragendorff's	Reagent Folin	Vanilin– H_2SO_4	Anisaldehyde– H_2SO_4	Comment
				Observation				
PR–WE1	83	-	Green (++)	-	Blue (+)	Green (+)	-	Phenol
PR–WE2	58	-	Yellow (+)	-	Blue (+)	Red (+)	-	Phenol

Indication for Intensity of colour:

+++   =   Strong

++   =   Moderate

+   =   Weak

-     =   No colour observed

The thin layer chromatography of extracts from *P. rubra* flower (Table 4.2–4.5), showed the presence of essential oils, phenols, flavonoids and terpenoids. The detection of these compounds was carried out using chemical reagents. The formation of blue spot with Folin–Ciocalteu reagent indicated the presence of phenols. The labeled compounds; PR–HE1 from the red flowers of PR–HE extract *P. rubra* using ethyl acetate : toluene (3: 7, v/v) as a solvent system (Table 4.2), PR–CE1 and PR–CE2 from the red flowers of PR–CE extract *P. rubra* by using methanol: chloroform (1: 9, v/v) as a solvent system (Table 4.3), the labeled compounds; PR–ME1, PR–ME2, PR–ME3, PR–ME4, PR–ME5, PR–ME6 and PR–ME7 from the red flowers of PR-ME extract *P. rubra* by using butanol: acetic acid: water (4: 5: 1, v/v) as a solvent system (Table 4.4), the labeled compounds PR–WE1 and PR–WE2 from the red flowers of PR–WE extract *P. rubra* by using butanol: acetic acid: water (3: 1: 1, v/v) as a solvent system (Table 4.5) were detected as phenol.

The presence of terpenoids was carried out using vanillin–sulphuric acid reagent with the formation of purple spot whereas the formation of red spots or other colour indicated the presence of phenols and flavonoids The labeled compound; PR–HE1 from the red flowers of PR–HE extract *P. rubra* by using toluene: ethyl acetate (7: 3, v/v) as a solvent system (Table 4.2), the labeled compound; PR–CE1 PR–CE2 and PR–CE3 from the red flowers of PR-CE extract *P. rubra* by using methanol: chloroform (1: 9, v/v) as a solvent system (Table 4.3), the labeled compounds; PR–ME1, PR–ME2, PR–ME3, PR–ME4, PR–ME5 and PR–ME7 from the red flowers of PR–ME extract *P. rubra* by using butanol: acetic acid: water (4: 5: 1, v/v) as a solvent system (Table 4.4) showed the presence of phenols and flavonoid whereas PR–ME6 showed the presence of terpenoids. The labeled compounds PR–WE1 and PR–WE2 from the red flowers of PR–WE extract

P. rubra by using using butanol: acetic acid: water (3: 1: 1, v/v) as a solvent system (Table 4.5) were detected as phenol and flavonoids.

Anisaldehyde–sulphuric acid reagent was carried out to all extracts of *P. rubra* red flower and the result showed the formation of blue spot indicated the presence of essential oils, whereas the red colour spots indicated the presence of flavonoid compound. The labeled compound; PR–HE1 from the red flowers of PR–HE extract *P. rubra* by using toluene: ethyl acetate (7: 3, v/v) as a solvent system (Table 4.2) was detected as essential oil, the labeled compound; PR–CE3 from the red flowers of PR–CE extract *P. rubra* by using methanol: chloroform (1: 9, v/v) as a solvent system (Table 4.3) and the labeled compounds; PR–ME3, PR–ME4, PR–ME5, and PR–ME7 from the red flowers of PR–ME extract *P. rubra* by using butanol: acetic acid: water (4: 5: 1, v/v) as a solvent system (Table 4.4),) were detected as flavonoid.

Dragendroff reagent was carried out to all extracts of *P.rubra* red flower and the TLC profile indicated no formation of orange spots which could be describde that there are no alkaloids in the red flower of *P.rubra*.

b. Liquid Chromatography Mass Spectrometry combined with Mass Spectrometry (LCMS/MS)

Liquid Chromatography Mass Spectrometry combined with Mass Spectrometry (LCMS/MS) was used to determine the chemical compounds present in extract of red flowers of *P.rubra*. The methanol (PR−ME) and water (PR−WE) extracts have been fully screen with AB Sciex 3200QTrap LCMS/MS and fully scan with MS/MS data collection.

i. PR−ME extract
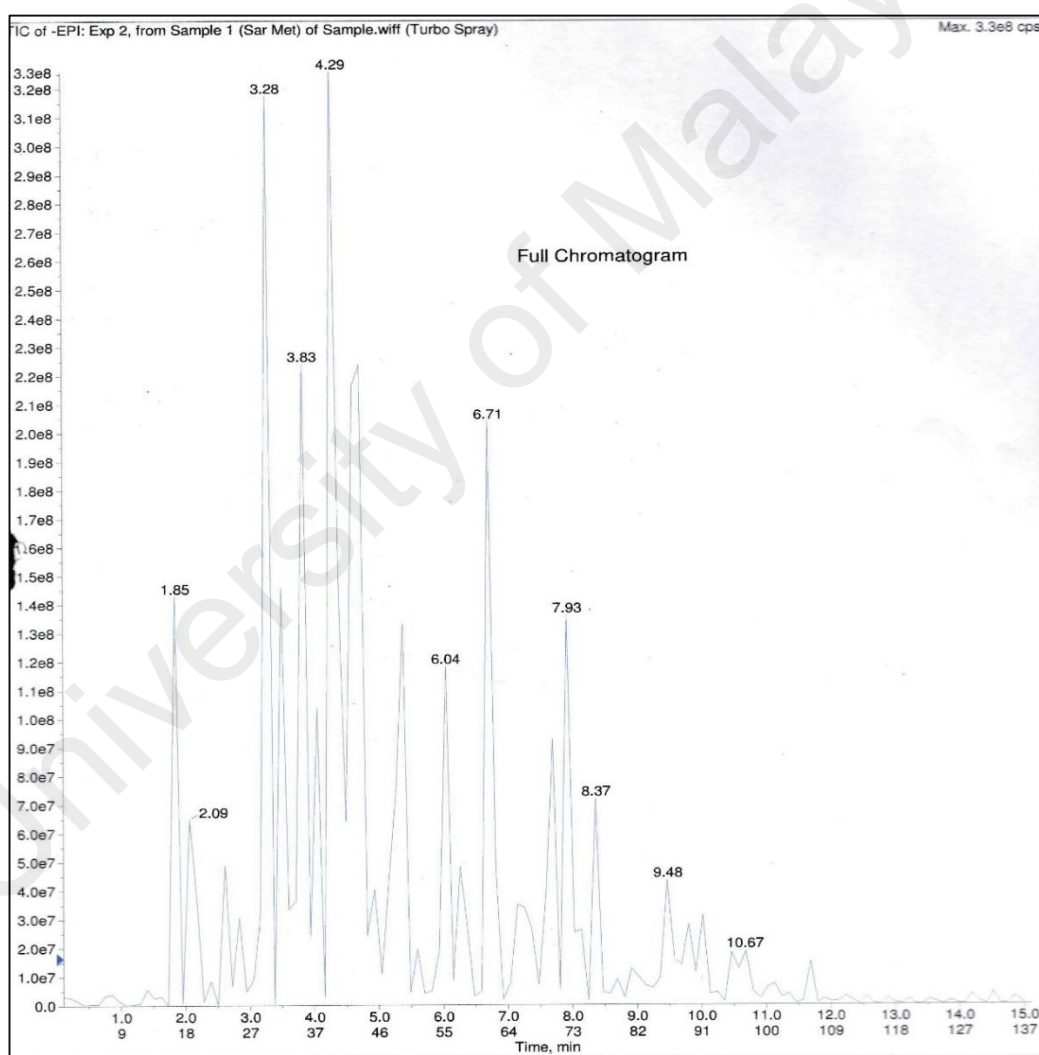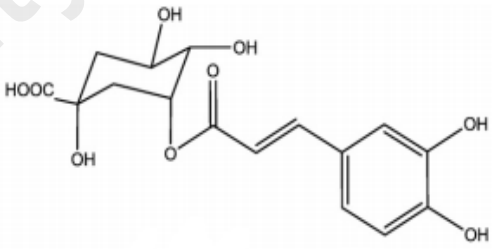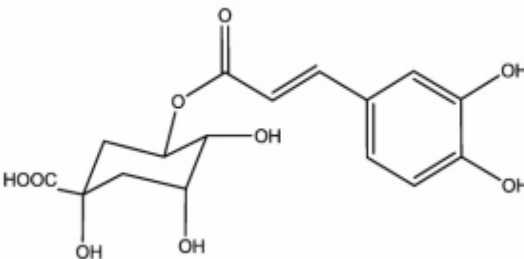
Figure 4.1. LCMS/MS Full Chromatogram of PR−ME extract from red flower of
P. rubra

Full LCMS chromatogram of PR–ME shows 11 peaks separated at different time of 1.85', 2.09', 3.28', 3.83', 4.29', 6.04', 6.71', 7.93', 8.37', 9.48', and 10.67' (Figure 4.1). 11 phytochemical compounds were detected in PR–ME extract of red flower *P.rubra* by LCMS/MS which comprised of 3–O–caffeoylquinic acid, 5–O–caffeoylquinic acid, chlorogenic acid, citric acid, kaempferol–3–O–glucoside, kaempferol–3–rutinoside, kaempferol, quercetin 3–O–α–L–arabinopyranside, quercetin, quinic acid and rutin.

Table 4.6 showed the chemical structure of phytochemical compounds which had been detected by LCMS/MS in PR–ME and these compounds were including of phenols and flavonoids.

Table 4.6. Chemical Structures of Phytochemical Compounds in PR–ME from Red flower of *P.rubra* Detected by LCMS/MS

Phytochemical Compound	Structure	Group
3–O–caffeoylquinic acid		Phenolic acid
5–O–caffeoylquinic acid		Phenolic acid
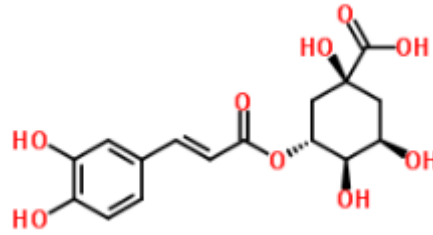
Chlorogenic acid		Phenolic acid
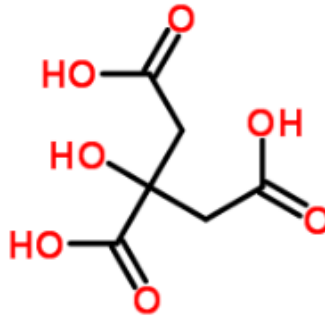
Citric acid		Organic acid

Kaempferol−3−O−glucoside (Astragalin)		Flavonoid
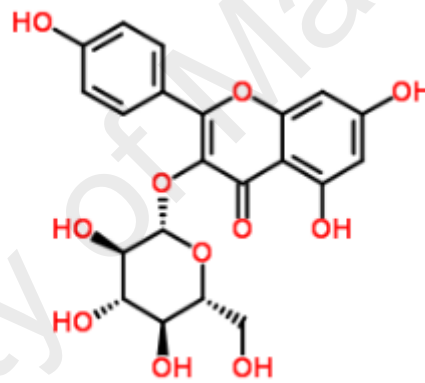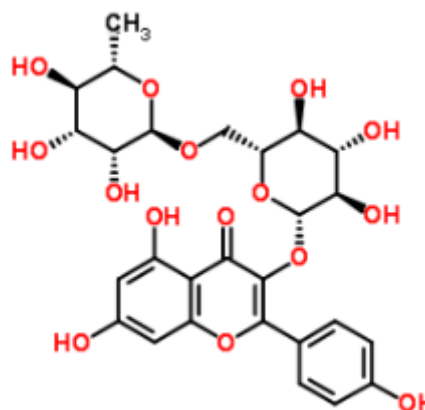
Kaempferol− 3 − rutinoside (Nicotiflorin)		Flavonoid
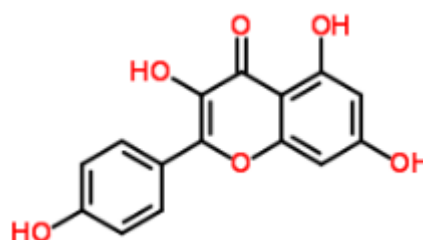
Kaempferol		Flavonoid

Quercetin 3− O − α − L − arabinopyranoside

Flavonoid
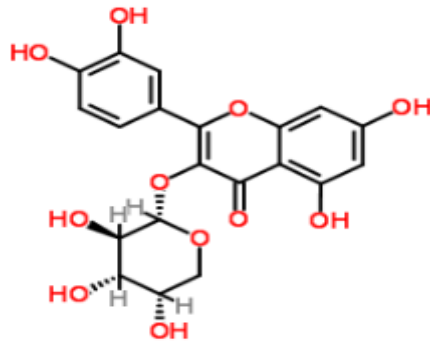
Quercetin
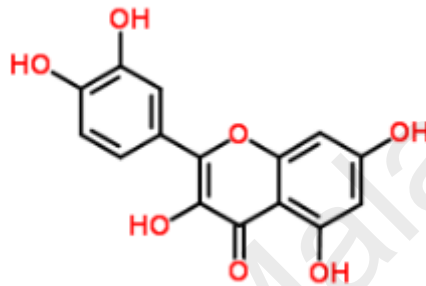
Flavonoid

Quinic acid

Phenolic acid
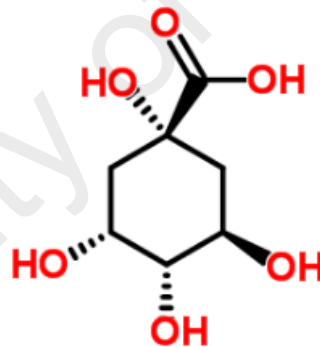
Rutin

Flavonoid
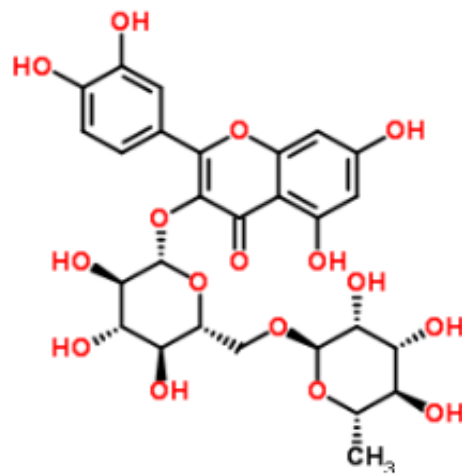
ii. PR–WE extract
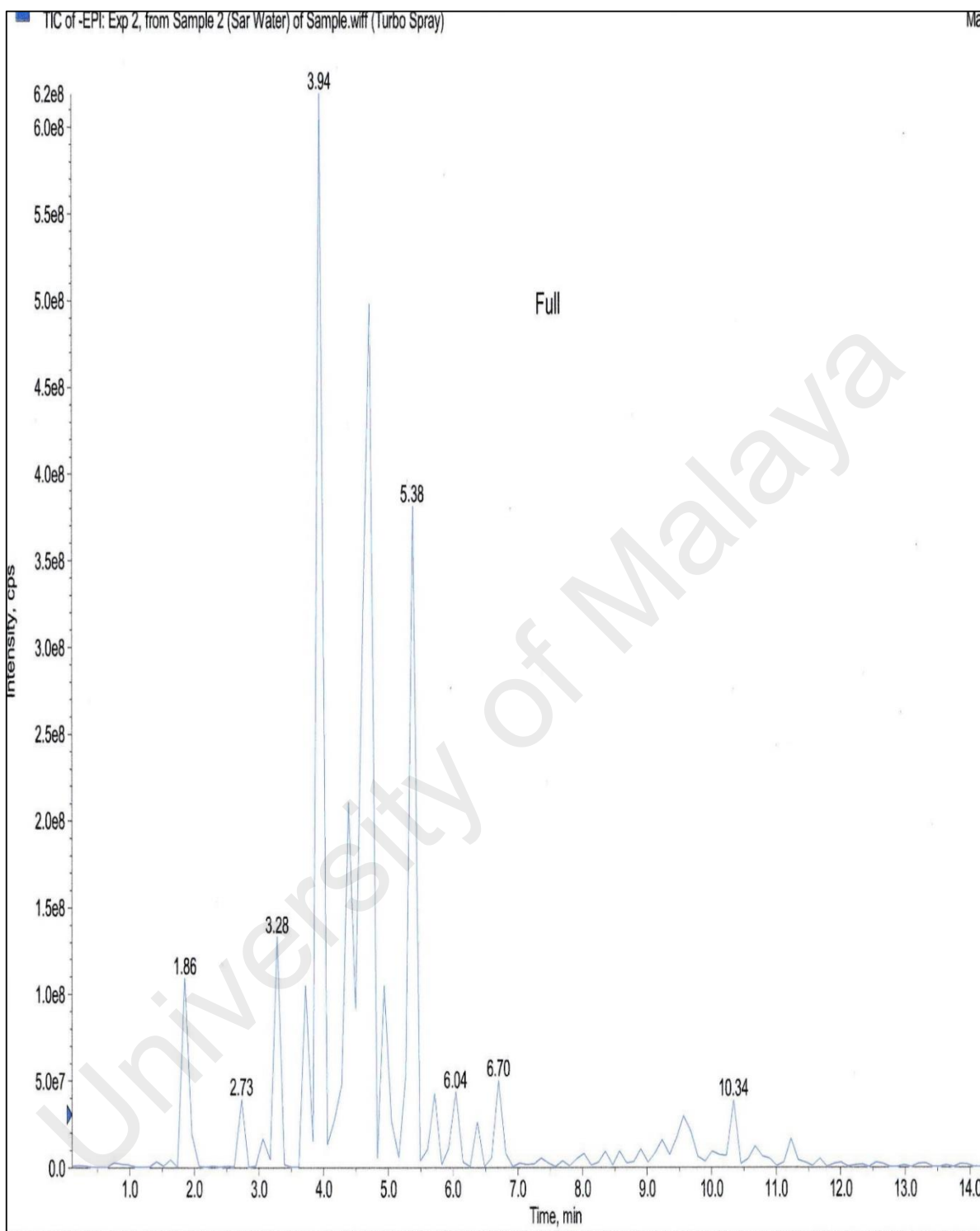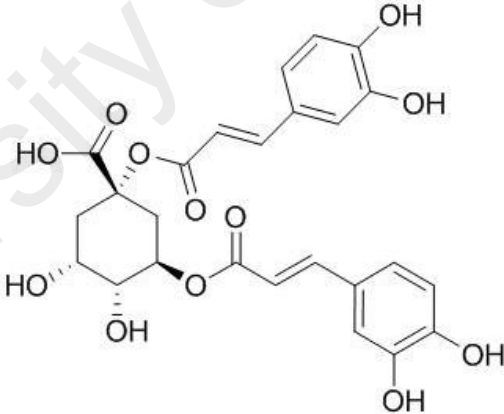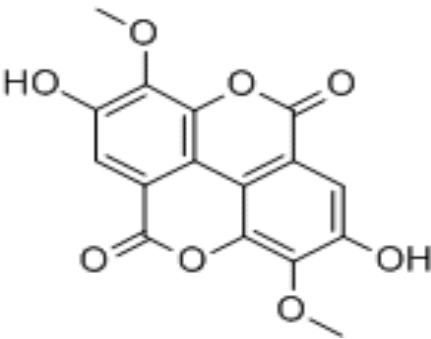
Figure 4.2. LCMS/MS Full Chromatogram of PR–WE extract from red flower of
P.rubra

Full LCMS chromatogram of water extract (PR−WE) shows 8 peaks separated at different time of 1.86', 2.73', 3.28', 3.94', 5.38', 6.04', 6.70' and 10.34' (Figure 4.2). 7 phytochemical compounds were detected in water extract from red flower *P.rubra* by LCMS/MS which comprised of 1, 3− dicaffeoquinic acid, 3, 3−di−O−methyl ellagic acid, 3−O−caffeyolquinic acid, kaempferol −3−O− glucoside, kaempferol−3−rutinoside, quercetin and quinic acid.

Table 4.7 showed the chemical structure of phytochemical compounds which had beed detected by LCMS/MS in PR−WE and these compounds were including of phenols and flavonoids.

Table 4.7. Chemical Structures of Phytochemical Compounds in PR−WE from Red flower of *P.rubra* Detected by LCMS/MS

Phytochemical Compound	Structure	Group
1,3−dicaffeoquinic acid		Phenolic acid
3,3−di−O−methylellagic acid		Phenolic acid
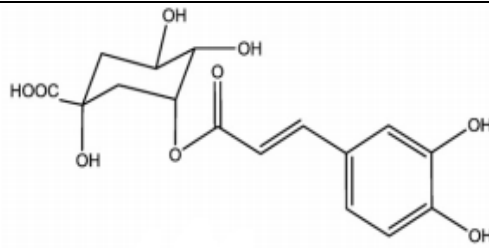
3−O−Caffeoylquinic acid		Phenolic acid
Kaempferol−3−O− glucoside (Astragalin)		Flavonoid
Kaempferol−3− rutinoside (Nicotiflorin)		Flavonoid
Quercetin		Flavonoid
Quinic acid		Phenolic acid
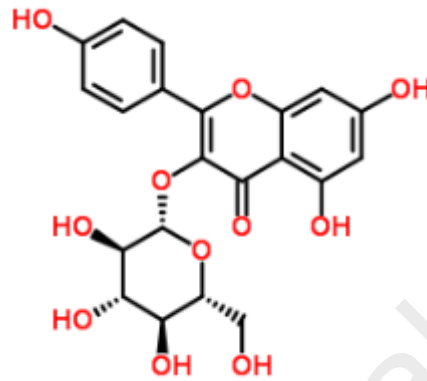
85
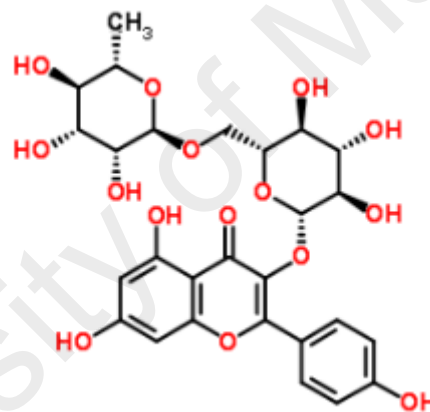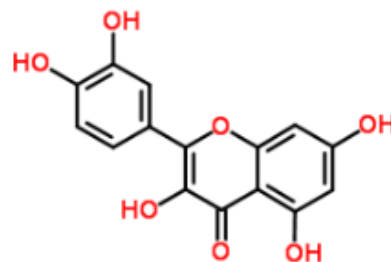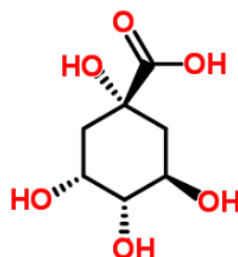
Table 4.8 Summary of LCMS/MS Profile of *P. rubra* Red flowers extracts

Extract of *P. rubra*	Tentative ID	Reference Figure (Refer to Appendices)	Retention Time
PR–ME	3−Caffeyolquinic acid	Figure 8.01	2.09'
	5−Caffeoquinic acid	Figure 8.02	Between 2.09' and 3.28'
	Chlorogenic acid	Figure 8.03	Between 3.28' and 3.83'
	Citric acid	Figure 8.04	Between 2.09' and 3.28'
	Kaempferol−3−O−glucoside	Figure 8.05	Between 4.29' and 6.04'
	Kaempferol−3−rutinoside	Figure 8.06	Between 4.29' and 6.04'
	Kaempferol	Figure 8.07	6.71'
	Quercetin 3−O−α−L−arabinopyranoside	Figure 8.08	Between 3.83' and 4.29'
	Quercetin	Figure 8.09	6.04'
	Quinic acid	Figure 8.10	1.85'
	Rutin	Figure 8.11	4.29'
PR–WE	1,3− Dicaffeoquinic acid	Figure 8.12	Between 3.94' and 5.38'
	3,3−di−O−methylellagic acid	Figure 8.13	6.70'
	3−Caffeoylquinic acid	Figure 8.14	Between 3.28' and 3.94'
	Kaempferol−3−O−glucoside	Figure 8.15	Between 3.94' and 5.38'
	Kaempferol−3−rutinoside	Figure 8.16	Between 3.94' and 5.38
	Quercetin	Figure 8.17	6.04'
	Quinic acid	Figure 8.18	1.86'

4.3 Determination of Total Phenolic Contents from *P. rubra* flower extract

Total Phenolic Content (TPC) in different extracts of *P. rubra* flower were determined by Folin-Ciocalteu (FC) method using gallic acid as the standard. The absorbance values obtained at different concentrations of gallic acid was used for the construction of standard curve. TPC of the extracts was calculated from the regression equation of calibration curve (y=0.0019x + 0.0255, r^2=0.9906) and expressed as mg gallic acid equivalents (GAE) per gram of sample in dry weight (mg/g) (Figure 4.3). The values of TPC in hexane (PR−HE), chloroform (PR−CE), methanol (PR−ME) and water (PR−WE) extracts of *P. rubra* flower are presented in Table 4.9. The TPC range from 2.210 ± 0.764 to 184.632 ± 0.765 mg equivalent gallic acid/g dry extract. The highest TPC have been detected in methanol (PR−ME) extract which is 184.63 ± 0.77 mg equivalent gallic acid /g dry extract.
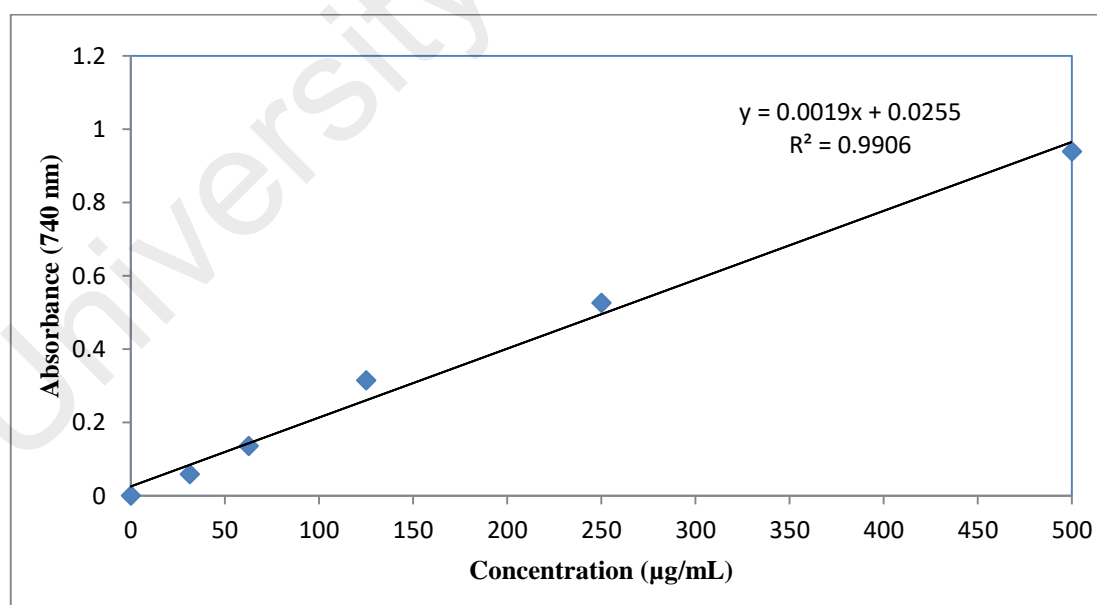
Figure 4.3. Standard curve of gallic acid

4.4 Determination of Total Flavonoid Contents from *P. rubra* flower extracts

The Total Flavonoid Content (TFC) in different extracts were measured by using aluminium chloride method and used quercetin as standard equivalent. The result was expressed as mg quercetin equivalents (QAE) per gram of sample in dry weight (mg/g). The standard curve equation was obtained was y=0.0005x + 0.0294, r2=0.9819) as presented in Figure 4.4. The values of TFC in hexane (PR–HE), chloroform (PR–CE), methanol (PR–ME) and water (PR–WE) extracts of *P. rubra* flower are presented in Table 4.9. The TFC range from 1.2± 1.16 to 203.2 ± 1.76 mg equivalent quercetin/g dry extract. The highest TFC have been detected in PR – ME extract which is 203.20 ± 1.76 mg equivalent quercetin /g dry extract.
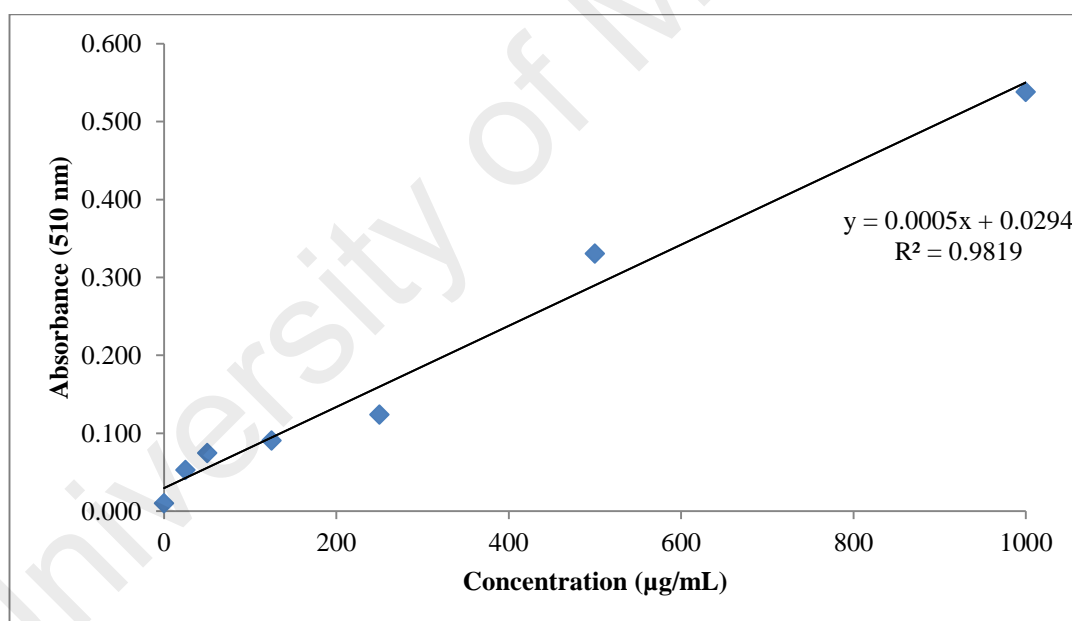
Figure 4.4. Standard curve of quercetin

TABLE 4.9. TPC and TFC Values of *P. rubra* flower extracts. Each values is represented as mean ± SE (n = 3). The means with different lower case letters (a, b, c, and d) in the same column are significantly different at P < 0.05 (ANOVA, followed by Duncan's multiple comparison test).

Extracts of *P. rubra*	Total Phenolic Content (mg GAE)/g dry extract	Total Flavonoid Content (mg QE)/ g dry extract
PR-HE	2.21 ± 0.76a	1.20 ± 1.16a
PR-CE	66.21 ± 1.27b	119.20 ± 2.40b
PR-ME	184.63± 0.77c	203.20 ± 1.76c
PR-WE	110.79 ± 1.07d	163.20 ± 1.33d

4.5 Antioxidant Activity Assay

The purpose of this study was to evaluate the potential of *P. rubra* red flower extracts by using six different assays.

4.5.1   2, 2–diphenyl–1–picrylhydrazyl (DPPH) Radical Scavenging Activity Assay

The potential antioxidant activity of PR–HE, PR–CE, PR–ME and PR–WE extracts of *P. rubra* flowers were determined on the basis of scavenging activity against the 2, 2 – diphenyl – 1 – picrylhydrazyl (DPPH) radicals. The radicals were characterized by a typical deep purple colour and maximum absorbance in the range of 515–520 nm.

Ascorbic acid was chosen in this study since it is a powerful antioxidant and the synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytolune (BHT), have restricted use in foods as these synthetic antioxidants are suspected to be carcinogenic (Madhavi *et al.* 1996).

The scavenging effect of the ascorbic acid on DPPH radicals was presented in Table 4.10.

Table 4.10. Percentage of Inhibition of DPPH radical by standard reference, ascorbic acid. Percentage of inhibition are expressed as mean ± SE, (n=3)

Concentration of Ascorbic acid (µg/mL)	Percentage of DPPH inhibition (%)
15.63	36.23 ±0.003
31.25	52.79 ± 0.002
62.5	72.62 ± 0.002
125	80.66 ±0.004
250	85.57 ± 0.003

The radical scavenging activity of ascorbic acid increased with increasing concentrations. The percentage of inhibition of ascorbic acid ranged between 36.23 % at 15.63 µg/mL to 85.57 % at 250 µg/mL. The percentage of inhibition of ascorbic acid at concentration 31.25, 62.5 and 125 µg/mL were 52.79 %, 72.62 % and 80.66 %, respectively.

The result of DPPH radical scavenging assay for PR–HE, PR–CE, PR–ME and PR–WE extracts of *P. rubra* red flower at concentration 15.63, 31.25, 62.5, 125 and 250 µg/mL are shown in Table 4.11.

Table 4.11. Radical scavenging activity of the hexane (PR–HE), chloroform PR–CE), methanol (PR–ME) and water (PR–WE) extracts of *P. rubra* flower in DPPH Radical Scavenging assay. Percentage of Inhibition (%) of extracts are expressed as mean ± SE, (n=3)

Concentration of Sample Extracts (µg/mL)	Percentage of DPPH Inhibition (%)			
	PR–HE extract	PR–CE extract	PR–ME extract	PR–WE extract
15.63	10.16 ± 0.002	14.26 ± 0.002	22.62 ± 0.001	18.53 ± 0.003
31.25	16.89 ± 0.002	18.85± 0.002	35.25 ± 0.002	24.43 ± 0.002
62.5	20.16± 0.002	23.77 ±0.003	52.62 ± 0.003	32.95 ± 0.004
125	22.62 ± 0.002	24.43 ± 0.01	63.44 ± 0.001	44.26 ± 0.004
250	29.34 ± 0.007	42.46 ± 0.002	70.98 ± 0.003	54.10 ± 0.002

The percentage of inhibition of DPPH radical scavenging activity of PR–HE, PR–CE, PR–ME and PR–WE extracts of *P. rubra* flowers increased with increasing concentration of the extracts. Among the four extracts, PR–ME extract exhibited the highest inhibition of DPPH scavenging activity with 70.98 % inhibition at the highest concentration tested, 250 μg/mL, followed by PR–WE, PR–CE and PR–HE extracts with 54.10 %, 42.46 % and 29.34 % inhibition of DPPH scavenging activity at the same concentration, respectively.

Figure 4.5 illustrated the dose response curve of DPPH radical scavenging activity of reference standard ascorbic acid, PR–HE, PR–CE, PR–ME and PR–WE extracts of *P. rubra* flower.
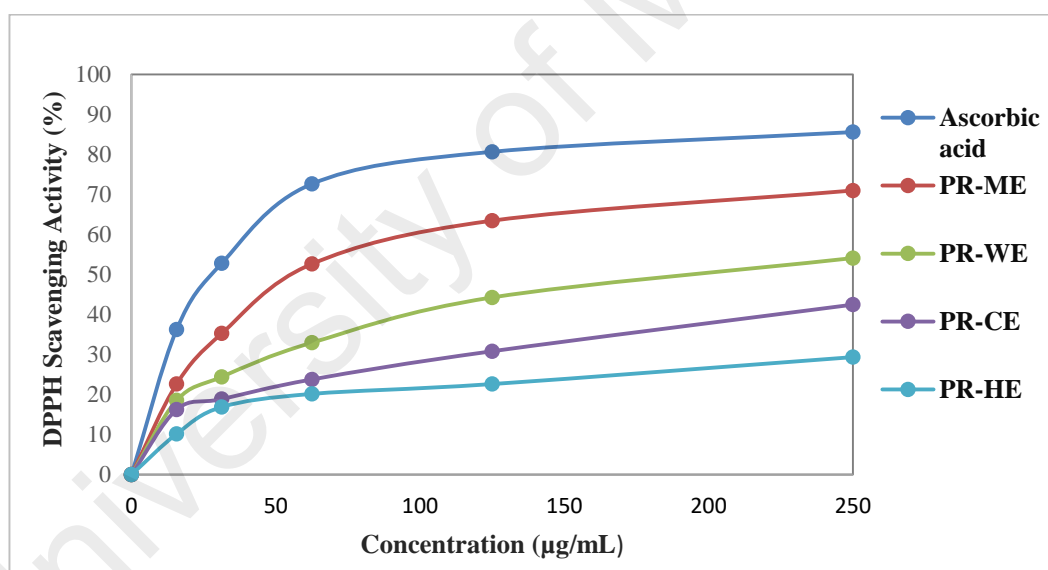
Figure 4.5 DPPH radical scavenging capacity of flowers extracts from *P. rubra*. All analyses were performed in triplicate. Results expressed as mean ±SE (n=3)

In comparison with the positive reference standard ascorbic acid, the percentage inhibition of DPPH radical activity of the PR–ME extract was approximately 1.3 fold lesser compared to the ascorbic acid. From the Table 4.20, the IC_{50} of PR–ME extract is 59.39 μg/mL while the IC_{50} standard ascorbic acid is 29.6 μg/mL. Nevertheless, there are no significant difference of DPPH scavenging activity between PR–ME extract

and standard ascorbic acid (p<0.05). On the other hand, PR–WE extract showed moderate scavenging activity at highest concentration tested which the IC_{50} value was 231.06 µg/mL but show no significant difference with PR–CE extract (p<0.05). The IC_{50} of PR–HE and PR−CE extract could not be determined at the concentration evaluated due to inhibition percentage of DPPH radical did not reach 50 % even at the highest concentration tested. The order of DPPH radical scavenging activity of extracts from *P. rubra* flowers was ascorbic acid > PR–ME> PR–WE> PR–CE>PR–HE.

4.5.2 Ferric Reducing Anti−Oxidant Power Assay

The purpose of reducing power assay is to evaluate the ability of *P. rubra* red flower extracts to reduce Fe^{3+} to Fe^{2+}.

The FRAP assay is based on the reduction, at low pH, of a colorless ferric complex (Fe^{3+}-tripyridyltriazine) to a blue-colored ferrous complex (Fe^{2+} – tripyridyltriazine) by the action of electron-donating antioxidants. The reduction is monitored by measuring the change of absorbance at 595 nm. Ferrous sulphate (Fe_2SO_4) was used as the reference standard. The FRAP value was evaluated according to the linear regression between reference standard solutions and absorbance at 595 nm and the results were estimated as mmol Fe^{2+}/g of dry extract. Standards of known Fe (II) concentrations ($FeSO4·7H_2O$) were run in triplicate using several concentrations between 200 and 1000 µg/mL (200, 400, 600, 800 and 1000 µg/mL). Figure 4.6 showed the linear regression of standard reference. In this assay, the reducing capacity of the extracts of *P.rubra* flower was calculated with reference to the reaction signal given by a Fe^{2+} solution.
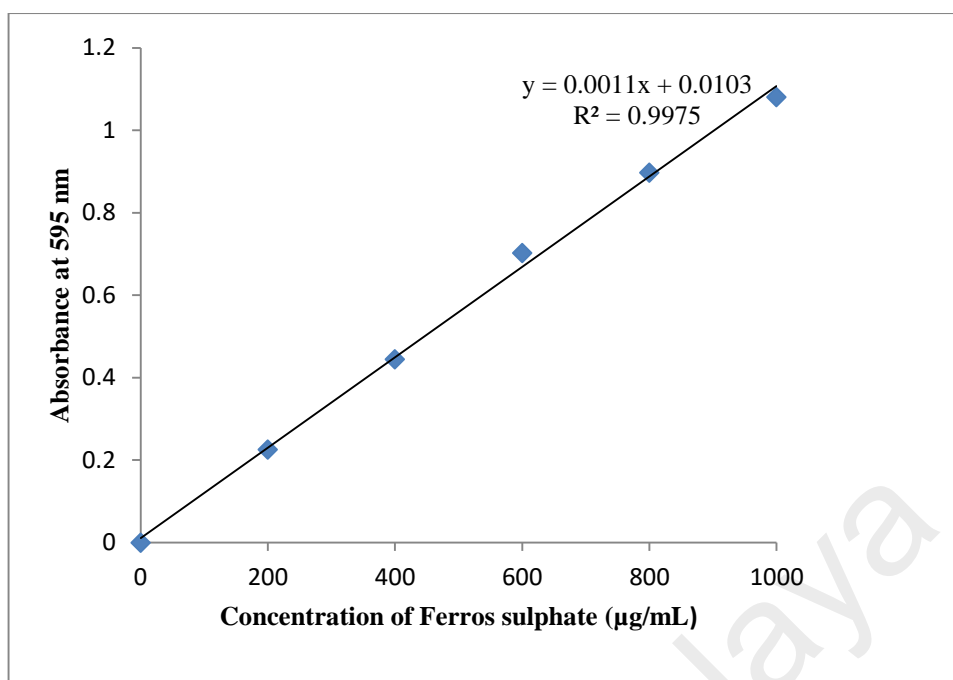
Figure 4.6. Standard curve of ferrous sulphate as a standard in the FRAP assay.

The ferric reducing power assay for PR–HE, PR–CE, PR–ME and PR–WE extracts of *P. rubra* red flower at concentrations 0.1 mg/mL and 1 mg/mL are shown in Figure 4.7. The result as depicted in Figure 4.7 and Table 4.20, were expressed as milimol ferrous iron equivalents per g of sample.

The reducing power of all four extracts increased with increasing concentrations. The order of FRAP value was PR–ME extract > PR–WE extract > PR–CE extract > PR–HE extract. In accordance with DPPH radical scavenging ability (Table 4.20), the highest FRAP value was detected in PR–ME extract of *P. rubra* flower with 4.95 ± 0.004 (mmol Fe^{2+}/g of dry extracts) followed by PR–WE extract of with 3.49 ± 0.03 (mmol Fe^{2+}/g of dry extracts) in *P. rubra* flower. The reducing ability of PR–HE with 0.22 ± 0.003 mmol Fe^{2+}/g of dry extracts was almost negligible. The moderate reducing power was detected in the medium polar solvent PR–CE extract with 0.97 ± 0.004 mmol Fe^{2+}/g of dry extracts.
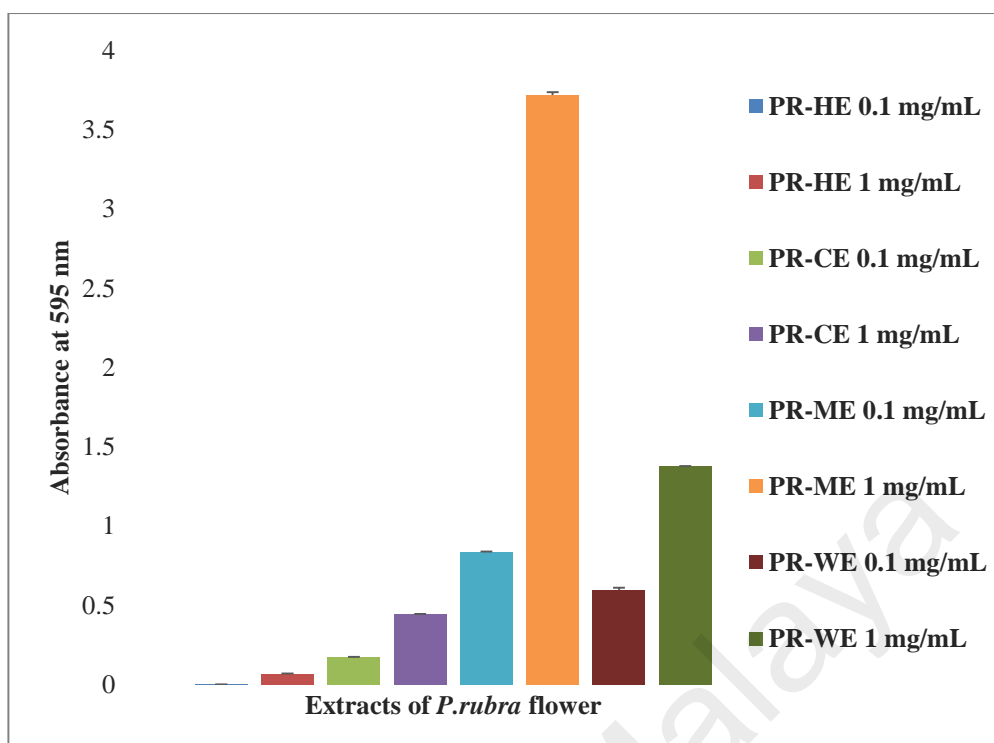
Figure 4.7 Ferric Reducing power of the *P. rubra* extracts

4.5.3 Metal Chelating Activity Assay

The purpose of metal chelating assay is to evaluate the ability of *P. rubra* red flower extracts to chelate ferrous ion and preventing the formation of ferrozine–Fe^{2+} complex.

EDTA (Ethy lenediaminetetra acetic acid) was used as the reference standard in the metal chelating assay. Table 4.12 shows the percentage of inhibition of Ferrozine–Fe^{2+} complex formation by standard EDTA at concentration of 15.63, 31.25, 62.5, 125 and 250 µg/mL.

Table 4.12. The Percentage of inhibition Ferrozine−Fe2+ complex formation by EDTA in metal chelating assay

Concentration of EDTA (μg/mL)	Percentage of Inhibition Ferrozine − Ferum^{2+} complex (%)
15.63	28.45 ± 0.004
31.25	45.19 ±0.002
62.5	55.65 ±0.005
125	71.97 ±0.009
250	92.89 ±0.003

The percentage of inhibition of Ferrozine − Fe^{2+} complex formation by EDTA increased with increasing concentrations. Very strong chelating activity was observed at highest concentration 250 μg/mL with 92.89 % chelating activity. The percentage of chelating activity at concentration of 15.63, 31.25, 62.5 and 125 μg/mL were 28.45 %, 45.12 %, 55.65 % and 71.97 %, respectively.

The result of metal chelating assay for PR−HE, PR−CE, PR−ME and PR−WE extracts of *P.rubra* red flower are shown in Table 4.13 and were obtained at concentrations 15.63, 31.25 62.5, 125 and 250 μg/mL.

Table 4.13. Metal chelating activities by hexane (PR−HE), chloroform (PR−CE), methanol (PR−ME) and water (PR−WE) extracts of *P.rubra* flower in metal chelating assay. All percentage of inhibition (%) are expressed as mean ± SE, (n=3)

Concentration of Extracts (μg/mL)	Percentage Inhibition of metal chelating (%)			
	PR−HE Extract	PR−CE Extract	PR−ME Extract	PR−WE Extract
15.63	4.60 ±0.004	7.53±0.007	14.64±0.01	7.11±0.01
31.25	13.81±0.004	8.79±0.008	23.01±0.004	14.64±0.003
62.5	18.83 ±0.001	21.76±0.0003	33.05±0.01	27.20±0.002
125	21.34±0.007	33.89±0.01	46.03±0.001	35.15±0.01
250	35.57±0.001	43.10±0.0003	67.36±0.01	50.21±0.003

The PR−ME extract was the highest metal chelator compared to other extracts with percentage of inhibition of 67.36 %, 46.03 %, 22.05 % and 23.01 % and 14.64 % at 250, 125, 62.5, 31.25 and 15.63 μg/mL, respectively. The metal chelating

activities of PR–WE extracts showed moderate activity with metal chelating activity inhibition of 50.21 % at highest concentration of 250 µg/mL then followed with PR–CE extract with metal chelating activity at 43.10 % at the same concentration. The PR–HE extract exhibited the weak metal chelating activity with only 35.57 % at concentration of 250 µg/mL.

Figure 4.8 illustrated the dose–response curves presenting the metal chelating activities of all four extracts of *P. rubra* flower and EDTA as a positive standard.
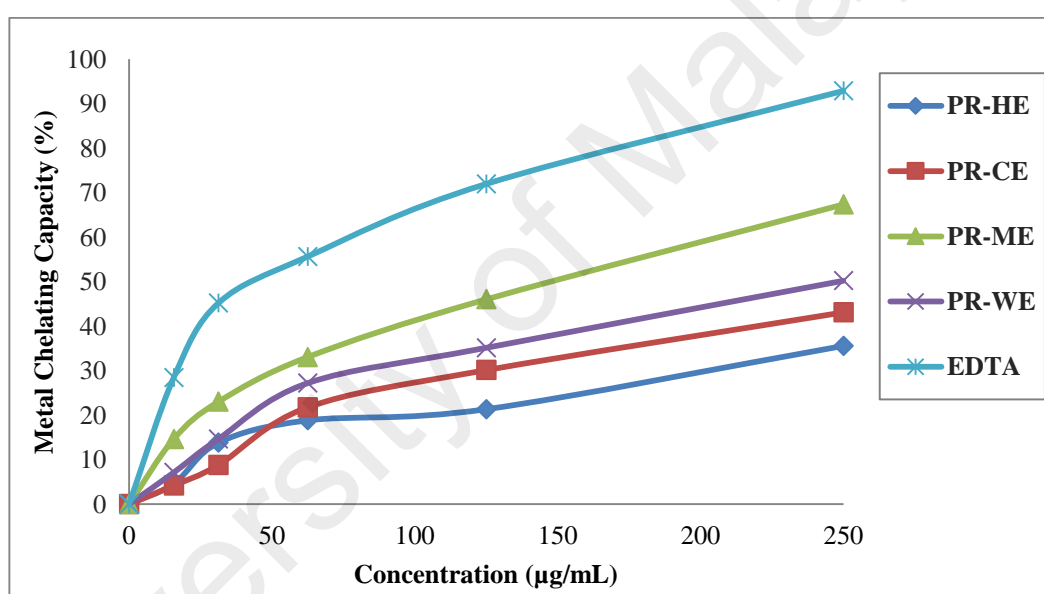
Figure 4.8. Metal chelating activity of *P. rubra* flower extracts and standard EDTA.

The percentage of inhibition of ferrozine –Fe^{2+} complex by all extracts and also including reference standard, EDTA were increased with increasing concentrations. EDTA possess the excellent of inhibition of Ferrozine– Fe^{2+} as it showed the best IC_{50} value which was 56.16 µg/mL. The IC_{50} value of PR–ME extract was 185.56 µg/mL while the IC_{50} value of PR–WE extract was 248.96 µg/mL as shown in Table 4.20. Moreover, the PR–ME extract possess highest significantly of metal chelating activity compared to other three extracts of flowers from *P.rubra* ($p<0.05$). The IC_{50} of PR–HE

and PR – CE extract could not be determined at the concentration evaluated due to inhibition percentage of metal chelating activity did not reach 50 % even at the highest concentration tested. The order of metal chelating activity of extracts from *P. rubra* flowers was EDTA> PR–ME> PR–WE> PR–CE>PR–HE.

4.5.4 Hydrogen Peroxide Radical Scavenging Activity Assay

The purpose of hydrogen peroxide scavenging assay is to evaluate the ability of extracts from *P.rubra* red flowers to scavenge hydrogen peroxide radical.

Ascorbic acid was used as the reference standard in the hydrogen peroxide radical scavenging assay. Table 4.14 shows the percentage of inhibition of hydrogen peroxide radical by standard ascorbic acid at concentration of 15.63, 31.25, 62.5, 125 and 250 µg/mL.

Table 4.14. The Percentage of inhibition hydrogen peroxide radical by ascorbic acid in hydrogen peroxide radical scavenging assay.

Concentration of Ascorbic acid (µg/mL)	Percentage of Inhibition of Hydrogen peroxide (%)
15.63	26.94±0.03
31.25	41.82±0.01
62.5	49.21±0.04
125	60.06±0.01
250	70.63±0.007

The radical scavenging activity of ascorbic acid increased with increasing concentrations. The percentage of inhibition of ascorbic acid ranged between 26.94 % at 15.63 µg/mL to 70.63 % at 250 µg/mL. The percentage of inhibition of ascorbic acid at concentration 31.25, 62.5 and 125 µg/mL were 41.82 %, 49.21 % and 60.06 %, respectively.

The result of hydrogen peroxide radical scavenging assay for PR−HE, PR−CE, PR−ME and PR−WE extracts of *P.rubra* red flower at concentration 15.63, 31.25, 62.5, 125 and 250 µg/mL are shown in Table 4.15.

Table 4.15. Radical scavenging activity of the hexane (PR−HE), chloroform (PR−CE), methanol (PR−ME) and water (PR−WE) extracts of *P. rubra* flower in Hydrogen peroxide Radical Scavenging assay. Percentage of Inhibition (%) of extracts are expressed as mean ± SE, (n=3)

Concentration of Extracts (µg/mL)	Percentage of Hydrogen peroxide Inhibition (%)			
	PR−HE extract	PR−CE extract	PR−ME extract	PR−WE extract
15.63	0.19±0.03	3.56±0.04	6.27±0.007	13.38±0.01
31.25	0.28±0.07	8.05±0.009	18.71±0.02	20.58±0.02
62.5	6.92±0.003	16.00±0.04	26.29±0.02	25.54±0.01
125	12.82±0.005	19.27±0.04	41.25±0.02	32.65±0.02
250	17.03±0.007	27.78±0.036	50.33±0.008	44.99±0.017

The percentage of inhibition of hydrogen peroxide radical scavenging activity of PR−HE, PR−CE, PR−ME and PR−WE extracts of *P.rubra* flowers increased with increasing concentration of the extracts. Among the four extracts, PR−ME extract exhibited the highest percentage of hydrogen peroxide scavenging activity with 50.33 % inhibition at concentration of 250 µg/mL followed by PR−WE, PR−CE and PR−HE extracts with 44.99 %, 27.78 % and 17.03 % at the same concentration, respectively.

Figure 4.9 illustrated the dose−response curve of hydrogen peroxide radical scavenging activity of reference standard ascorbic acid, PR−HE, PR−CE, PR−ME and PR−WE extracts of *P. rubra* flower.
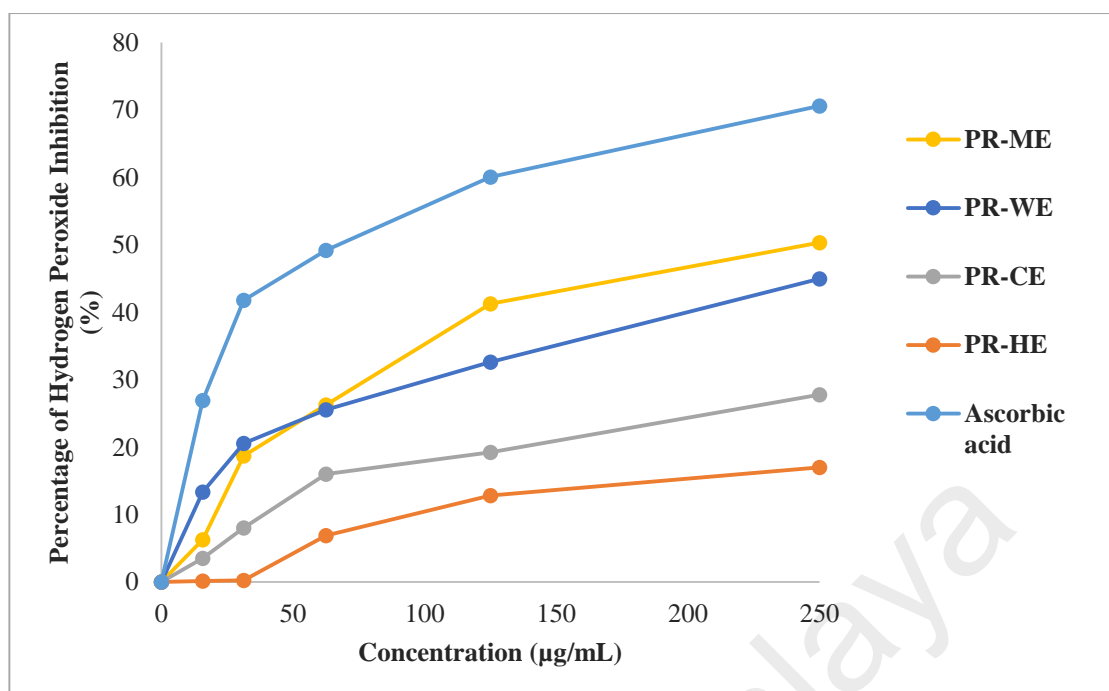
Figure 4.9. Hydrogen peroxide scavenging activity of *P. rubra* flower extracts and standard ascorbic acid.

In comparison with the positive reference standard ascorbic acid, PR–ME extract of *P. rubra* flower showed lower percentage inhibition of hydrogen peroxide activity at all concentrations. The IC_{50} of PR–ME extract is 248.38 µg/mL as shown while the IC_{50} standard ascorbic acid was 104.07 µg/mL as shown in Table 4.20. The IC_{50} of PR–HE, PR–CE and PR–WE extracts could not be determined at the concentration evaluated due to inhibition percentage of hydrogen peroxide radical did not reach 50 % even at the highest concentration tested. Nevertheless, there are no significant difference ($p < 0.05$) of hydrogen peroxide scavenging activity between PR–ME extract with the standard, ascorbic acid as well as with PR–WE extract. The order of hydrogen peroxide scavenging activity of extracts from *P.rubra* flowers was ascorbic acid > PR–ME > PR–WE > PR–CE > PR–HE.

4.5.5 Nitric Oxide Radical Scavenging Activity Assay

The purpose of nitric oxide scavenging assay is to evaluate the ability of *P. rubra* red flower extracts to scavenge the nitric oxide radical and compete with oxygen leading to reduced production of nitrite ions. Nitric oxide scavenging capacity is determined by the decrease in the absorbance at 546 nm.

Curcumin was used as the reference standard in the nitric oxide scavenging assay. Table 4.16 shows the percentage of inhibition of nitric oxide radical by standard curcumin at concentration of 15.63, 31.25, 62.5, 125 and 250 µg/mL.

Table 4.16. The Percentage of inhibition nitric oxide radical by curcumin in nitric oxide radical scavenging assay.

Concentration of Curcumin (µg/mL)	Percentage of Inhibition Nitric Oxide radical (%)
15.63	48.05 ±0.01
31.25	62.99 ±0.01
62.5	73.70 ±0.01
125	82.47 ±0.01
250	87.99 ±0.01

The percentage of inhibition of nitric oxide radical by curcumin increased with increasing concentrations. The strong scavenging activity was observed at highest concentration tested with 87.99 % chelating activity. The percentage of scavenging activity at concentration of 15.63, 31.25, 62.5 and 125 µg/mL were 48.05 %, 62.99 %, 73.70 % and 82.47 %, respectively.

The result of nitric oxide radical scavenging activity assay for PR–HE, PR–CE, PR–ME and PR–WE extracts of *P. rubra* red flower are shown in Table 4.17 and were obtained at concentrations 15.63, 31.25, 62.5, 125 and 250 µg/mL.

Table 4.17. Nitric Oxide scavenging activities by hexane (PR–HE), chloroform (PR–CE), methanol (PR–ME) and water (PR–WE) extracts of *P. rubra* flower in nitric oxide scavenging assay. All percentage of inhibition (%) are expressed as mean ± SE, (n=3)

Concentration of Extracts (µg/mL)	Percentage Inhibition of nitric oxide radical (%)			
	PR-HE Extract	PR-CE Extract	PR-ME Extract	PR-WE Extract
15.63	17.53±0.02	25.65±0.01	30.20±0.003	28.90±0.01
31.25	26.62±0.004	33.77±0.004	37.01±0.01	40.58±0.01
62.5	31.49±0.004	38.31±0.004	50.33±0.01	42.86±0.01
125	35.39±0.004	40.58±0.01	62.01±0.01	53.57±0.002
250	37.99±0.004	44.16±0.003	69.48±0.01	55.52±0.01

The PR–ME extract shown the highest scavenging activity against nitric oxide radical compared to other three extracts with percentage of inhibition of 69.48 %, 62.01 %, 50.33 % and 37.01 % and 30.20 % at 250, 125, 62.5, 31.25 and 15.63 µg/mL, respectively. The PR–WE extract exhibited moderate scavenging activity against nitric oxide radical with percentage of inhibition of 55.52 %, 53.57 %, 42.86 %, 40.58 % and 28.90 % at 250, 125, 62.5, 31.25 and 15.63 µg/mL, respectively. The PR–HE and PR–CE extract exhibited the weak nitric oxide scavenging activity with only 37.99 % and 44.16 % at concentration of 250 µg/mL, respectively. The nitric oxide scavenging activities of PR – WE extract showed moderate activity with scavenging activity of 55.52 % at concentration of 250 µg/mL.

Figure 4.10 illustrated the dose–response curves presenting the scavenging activities of all four extracts of *P. rubra* flower and curcumin as a positive standard against nitric oxide radical.
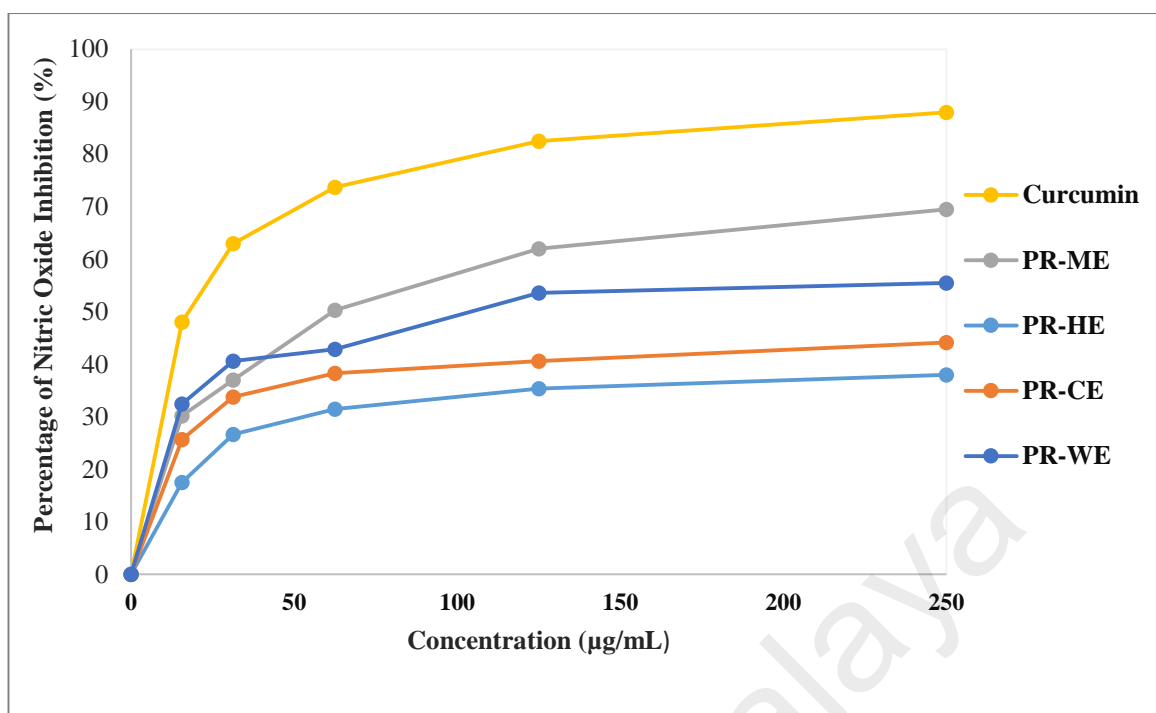
Figure 4.10. Nitric Oxide Scavenging activity of *P. rubra* flower extracts and standard Curcumin.

In comparison with the positive reference standard curcumin, PR‑ME extract *of P. rubra* flower showed lower percentage of nitric oxide inhibition at all concentrations. The IC_{50} of PR‑ME extract is 62.10 μg/mL as shown while the IC_{50} standard curcumin was 24.81 μg/mL as shown in Table 4.20. Nevertheless, PR‑ME extract showed the significant higher of nitric oxide scavenging activity compared the other three extracts of flower from *P. rubra* (p<0.05). The PR‑WE extract showed moderate scavenging activity which was 116.67 μg/mL. The IC_{50} of PR–HE and PR–CE extract could not be determined at the concentration evaluated due to inhibition percentage of nitric oxide radical did not reach 50 % even at the highest concentration tested. The order of nitric oxide scavenging activity of extracts from *P. rubra* flowers was curcumin > PR–ME > PR–WE > PR–CE > PR–HE.

4.5.6   Superoxide Radical Scavenging Activity Assay

The purpose of superoxide radical scavenging assay is to determine the capability of *P. rubra* red extracts to scavenge superoxide anion derived from dissolved oxygen by PMS–NADH coupling reaction reduces NBT in this system. The inhibition of superoxide radicals was measured in absorbance at 570 nm.

Ascorbic acid and quercetin were used as the reference standard in the superoxide radical scavenging assay. Table 4.18 shows the percentage of inhibition of superoxide radical by standard quercetin and ascorbic acid at concentration of 15.63, 31.25, 62.5, 125 and 250 µg/mL.

Table 4.18. The Percentage of superoxide inhibition by quercetin and ascorbic acid in superoxide radical scavenging Assay

Concentration of Quercetin (µg/mL)	Percentage of Superoxide Inhibition (%)	Concentration of Ascorbic acid (µg/ml)	Percentage of Superoxide Inhibition (%)
15.63	23.41 ±0.01	15.63	27.13±0.01
31.25	29.77 ±0.004	31.25	44.34±0.004
62.5	49.61 ±0.01	62.5	61.86±0.01
125	64.34 ±0.01	125	78.14±0.01
250	74.88 ±0.003	250	82.48±0.004

The percentage of superoxide radical inhibition by ascorbic acid and quercetin increased with the increasing concentrations. As a standard, ascorbic acid exhibited better scavenging activity of superoxide anions compared with standard, quercetin. Ascorbic acid showed good percentage of superoxide radical scavenging activities with 82.48 % at highest concentration tested, 250 µg/mL. However, quercetin only reached 74.88 % of superoxide anions scavenging activities at highest concentration tested. As depicted in Table 4.18, the percentage superoxide scavenging activity of ascorbic acid at concentration of 15.63, 31.25, 62.5 and 125 µg/mL of quercetin were

27.13 %, 44.34 %, 61.86 % and 78.14 %, respectively whereas quercetin at concentration

of 15.63, 31.25, 62.5 and 125 µg/mL were 23.41 %, 29.77 %, 49.61 % and 64.34 %,

The result of superoxide radical scavenging assay for PR−HE, PR−CE,

PR−ME and PR−WE extracts of *P. rubra* flower at concentration 15.63, 31.25, 62.5, 125

and 250 µg/mL are shown in Table 4.19.

Table 4.19. Superoxide radical scavenging activity of the hexane (PR−HE), chloroform (PR−CE), methanol (PR−ME) and water (PR−WE) extracts of *P. rubra* flower in superoxide radical scavenging assay. Percentage of inhibition (%) of extracts are expressed as mean ± SE, (n=3)

Concentration of Extracts (µg/ml)	Percentage of Superoxide Inhibition (%)			
	PR−HE extract	PR−CE extract	PR−ME extract	PR−WE extract
15.63	2.48 ±0.01	15.19±0.02	31.31±0.003	19.07±0.01
31.25	8.06 ±0.01	21.55±0.002	48.06±0.01	27.44±0.003
62.5	20.47 ±0.01	31.16±0.004	57.05±0.004	48.99±0.003
125	26.82 ±0.0003	38.45±0.01	68.53±0.001	63.41±0.003
250	36.74 ±0.001	50.08±0.01	79.54±0.01	70.85±0.004

From the Table 4.19, the percentage of superoxide radical scavenging

activity of PR−HE, PR−CE, PR−ME and PR−WE extracts of *P, rubra* flowers increased

with increasing concentration of the extracts. Among the four extracts, PR−ME extract

exhibited the highest scavenging activity of superoxide radical with 79.54 % inhibition at

250 µg/mL followed by PR−WE, PR−CE and PR−HE extracts with 70.85 %, 50.08 %

and 36.74 % inhibition of superoxide scavenging activity at the same concentration,

respectively.

Figure 4.11 illustrated the dose response curve of superoxide radical

scavenging activity of both reference standards including quercetin and ascorbic acid, as

well as sample extracts of *P.rubra* flower which are PR−HE, PR−CE, PR−ME and PR−

WE.
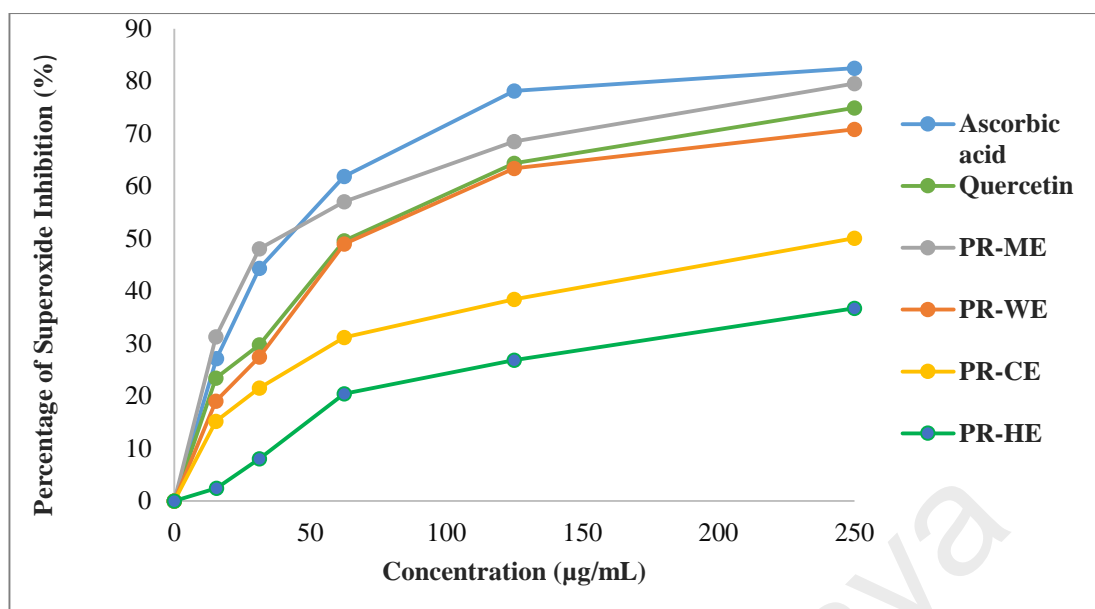
Figure 4.11. Superoxide scavenging activity of *P. rubra* flower extracts and standard, quercetin and ascorbic acid.

In comparison with the positive reference standard ascorbic acid, PR−ME extract of *P. rubra* flower showed lower percentage inhibition of superoxide radical at concentrations of 62.5, 125 and 250 µg/mL with 57.05 %, 68.53 % and 79.54 %, respectively whereas ascorbic acid presented 61.86 %, 78.14 % and 82.48 %, inhibition of superoxide radicals at the same concentration, respectively. As depicted in Figure 4.11, PR−ME extract presented slightly higher percentage of superoxide radical scavenging activity at the concentration of 15.53 µg/mL and 32.5 µg/mL with 31.32 % and 48.06 %, respectively compared to ascorbic acid showed percentage of superoxide scavenging activity with 27.13 % and 44.34 % at the same concentration, respectively. In addition, the IC_{50} of PR−ME extract was 54.77 µg/mL while the IC_{50} standard ascorbic acid was 50.52 µg/mL as shown in Table 4.20. Nevertheless, there are no significant difference of superoxide scavenging activity between the PR−ME extract with the standard, ascorbic acid ($p<0.05$).

Surprisingly, PR−ME extract exhibited significantly higher of superoxide scavenging activity compared to standard quercetin at all concentration tested ($p<0.05$).

Moreover, there are no significant difference of superoxide scavenging activity between PR – WE extract and the standard quercetin. From the Figure 4.11, PR – ME extract presented the percentage of superoxide scavenging activity with 79.54 % at the highest concentration tested, 250 µg/mL, whereas standard quercetin only reached percentage of superoxide scavenging activity with 74.88 % at the same concentration. According to Table 4.20, the IC_{50} of standard quercetin was 97.13 µg/mL compared to IC_{50} of PR–ME extract was 54.773 µg/mL.

Among the four extracts, PR–ME extract exhibited the significant highest scavenging activity of superoxide radical ($p<0.05$).The PR–WE extract showed moderate scavenging activity of superoxide followed with PR – CE extract at the highest concentration tested which showed 70.85 % and 50.08 %, respectively. The IC_{50} of PR–WE was 98.563 µg/mL while the IC_{50} of PR–CE extract was 249.611 µg/mL. The IC_{50} of PR–HE extract could not be determined at the concentration evaluated due to inhibition percentage of hydrogen peroxide radical did not reach 50 % even at the highest concentration tested. Therefore, the order of superoxide scavenging activity in present study was ascorbic acid > PR–ME extract > quercetin > PR–WE > PR–CE extract> PR–HE extract.

Table 4.20. IC$_{50}$ and FRAP values of antioxidant activities of *P. rubra* flower extracts. Each value in the table is represented as mean ± SE (n = 3). The means with different lower case letters (a, b, c, and d) in the same column are significantly different at P < 0.05 (ANOVA, followed by Duncan's multiple comparison test). ND: not detected.

Extracts / Positive Control	DPPH (IC$_{50}$ µg/mL)	FRAP (mmol Fe^{2+}/g extract)	Metal Chelating (IC$_{50}$ µg/mL)	Hydrogen Peroxide (IC$_{50}$ µg/mL)	Nitric Oxide (IC$_{50}$ µg/mL)	Superoxide (IC$_{50}$ µg/mL)
PR-HE	ND	0.220 ± 0.003 a	ND	ND	ND	ND
PR-CE	ND	0.973 ± 0.004 b	ND	ND	ND	249.61± 0.01 c
PR-ME	59.39± 0.003 a	4.95 ± 0.004 c	185.56 ± 0.01 a	248.38± 0.004 a,b,c	62.10 ± 0.01 a	54.77 ± 0.004 a,d
PR-WE	231.06 ± 0.002 b, c	3.5 ± 0.03 d	248.96 ± 0.003 b	ND	116.67± 0.002 b	98.56± 0.004 b, e
Ascorbic acid	29.6 ± 0.002 d	_	_	104.07 ± 0.01 a, b, c	_	50.52 ± 0.01 a, d
EDTA	_	_	56.157± 0.004 c	_	_	_
Curcumin	_	_	_	_	24.81± 0.01 c	-
Quercetin	_	_	_	_	_	97.14± 0.01 b, e

4.6. Xanthine Oxidase Inhibitory Activity in *in vitro* Model

The purpose of this study is to determine the capability of extracts from *P. rubra* red flowers to inhibit the activity of enzyme, xanthine oxidase (XO) in producing uric acid in *in vitro* model. XO inhibitory activity was assayed spectrophotometrically under aerobic conditions and the degree of enzyme inhibition was determined by measuring the increase in absorbance at 295 nm associated with uric acid formation.

Allopurinol, a known inhibitor of XO, was used as a positive reference standard due to prescription for many years of this drug to treat gout disease. Allopurinol has high capability to inhibit action of xanthine oxidase and catalyses the oxidation of xanthine and hypoxanthine into uric acid. Table 4.21 showed the percentage of inhibition of XO by standard allopurinol at concentration of 25, 50, 100 and 200 µg/mL.

Table 4.21. The percentage of inhibition of XO by standard reference, allopurinol in *in vitro* Assay. All percentage of inhibition (%) are expressed as mean ± SE, (n=3)

Concentration of allopurinol (µg/mL)	Percentage of XO inhibition (%)
25	77.15 ±0.71
50	80.93 ±0.16
100	86.63 ± 0.06
200	90.81 ± 3.47

The XO inhibition activity of allopurinol showed strong inhibition activity and this activity was increased with increasing concentrations. The percentage of XO inhibition of allopurinol ranged between 77.15 % at 25 µg/mL to 90.81 % at 200 µg/mL. The percentage of XO inhibition of allopurinol at concentration 50 µg/mL and 100 µg/mL were 80.93 % and 86.63 %, respectively.

The influenced of the *P. rubra* red flower extracts on XO activity evaluated by decreased production of uric acid, which was measured spectrophotometrically. The result of XO inhibitory activity assay for all extracts of *P. rubra* flower at concentration 25, 50, 100, and 200µg/mL are shown in Table 4.20.

Table 4.22. XO Inhibition Activitity of hexane (PR−HE), chloroform (PR−CE), methanol (PR−ME) and water (PR−WE) extracts of *P. rubra* flower *in vitro* assay. All percentage of inhibition (%) are expressed as mean ± SE, (n=3)

Concentration of Extracts (µg/mL)	Percentage of XO Inhibition (%)			
	PR−HE Extract	PR−CE Extract	PR−ME Extract	PR−WE Extract
25	29.95 ± 2.67	42.99 ± 1.83	52.27 ± 2.00	49.66 ± 3.15
50	39.93± 3.35	50.94± 5.28	68.84 ± 1.69	60.5 ± 2.00
100	50.8± 1.23	61.57 ± 3.2	79.89 ± 0.60	64.53± 4.29
200	61.21± 2.26	63.96± 2.76	84.39 ± 1.12	76.15 ± 2.69

Figure 4.12 illustrate the different effect of four different concentration of PR−HE, PR−CE, PR−ME and PR−WE extract of *P. rubra* flower compared to standard positive control, allopurinol in inhibition of xanthine oxidase enzyme. All the four extracts showed XO inhibitory activity in dose dependent manner. As shown in Figure 4.12, all extracts were capable to inhibited XO−induced superoxide formation at the highest concentration tested, 200 µg/mL. The order of XO inhibition activity in present study was allopurinol > PR−ME extract > PR−WE > PR−CE extract> PR−HE extract. From the Table 4.20, the percentage of inhibition of XO activity by PR−HE, PR−CE, PR−ME and PR−WE extracts of *P. rubra* flowers increased with increasing concentration of the extracts. Among the four extracts, PR−ME extract exhibited the highest inhibition of XO activity with 52.27 % inhibition at concentration of 25 µg/mL followed by PR−WE, PR−CE and PR−HE extracts with 49.66 %, 42.99 % and 29.95 % inhibition of XO activity at the same concentration, respectively. PR−ME extract also appeared as the strongest XO inhibitor compared to other four extracts with highest percentage of XO inhibition in

all concentration tested which was 68.84 % at concentration of 50 µg/mL, 79.89 % at concentration of 100 µg/mL and 84.39 % at 200 µg/mL.
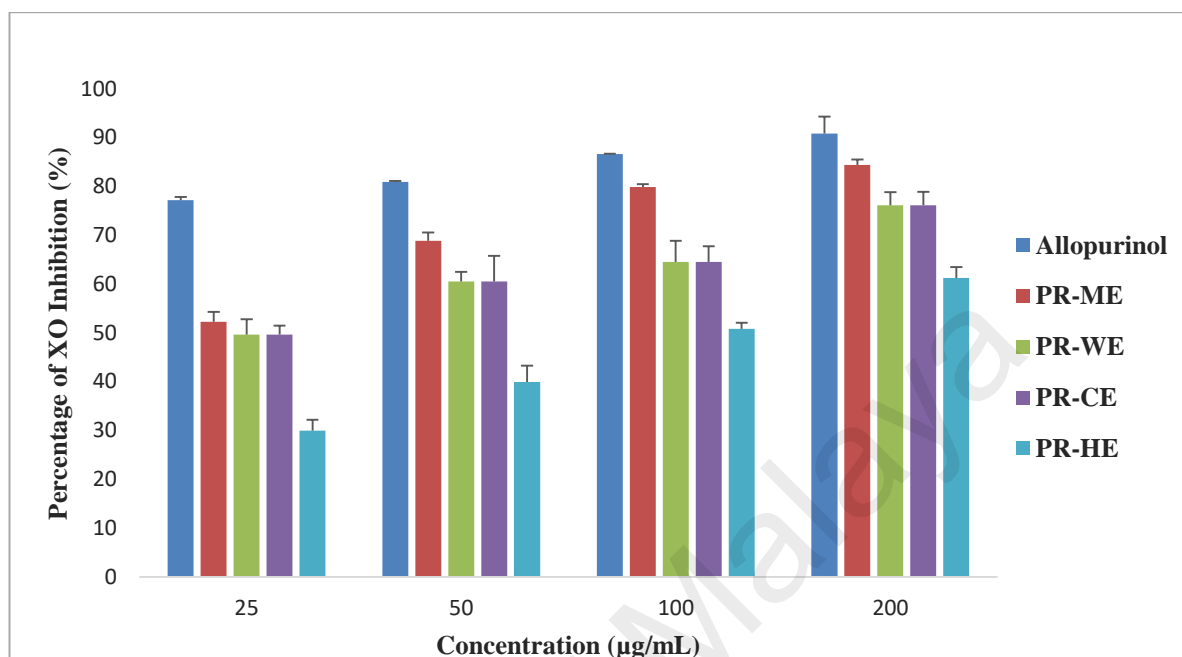
Figure 4.12. Percentage xanthine oxidase (XO) inhibitory activity of different extracts which are various concentrations from *P.rubra* flower *in vitro*. Each value is represented as mean ± S.E. of three separate experiments in triplicate measurements.

The positive control, allopurinol, showed the best IC_{50} value which was 16.20 µg/mL. As depicted in Figure 4.12, PR−ME extract exhibited the strongest XO inhibitory activity compared to other three extracts with the IC_{50} which was 23.91 µg/mL followed by PR−WE extract produced the IC_{50} value which was 41.32 µg/mL as shown in Table 4.23. PR−CE extract showed the moderate inhibition activity of XO enzyme which the IC_{50} value was 49.08 µg/mL (Table 4.23). The PR−HE extract showed the lowest inhibition activity of XO enzyme which the IC_{50} value was 98.43 µg/mL as shown in Table 4.23. From the present result of XO inhibitory activity in *in vitro*, PR–ME extract produced significantly highest inhibition activity than those other extracts. Thus, PR–ME extract of *P. rubra* flower was selected for the screening of uric acid clearance via

hyperuricemic rat model in *in vivo* and also xanthine oxidase inhibitory activity in serum and liver *in vivo*.

Table 4.23. IC$_{50}$ value of crude extracts from *P.rubra* flowers and standard reference, allopurinol in inhibition activity of XO enzyme

Extracts	IC$_{50}$ (µg/mL)
PR−HE	98.43 ± 1.23 a
PR−CE	49.08 ± 5.28 b
PR−ME	23.91 ± 2 c
PR−WE	41.32 ± 2 d
Allopurinol	16.20 ± 0.71 e

4.7 Xanthine Oxidase Inhibitory Activity in *in Vivo* Model

Acute toxicity study revealed the non-toxic nature of the extracts. There was no lethality or any toxic behaviours found at single dose selected until the end of the study period. The PR−ME extract of *P. rubra* red flower did not show any mortality and none of the orally administered rats in both sexes showed any visible symptoms of toxicity up to a dose of 4 g/kg body weight. In addition, at this single dose, there were no gross behavioral changes indicating high margins of safety.

4.71 Effect PR−ME Extract in Uric acid in *in Vivo* Model

The purpose of this study is to determine the anti-hyperuricemic activity or uric-acid clearance of PR−ME extract of *P. rubra* red flower in experimental rat model in *in vivo* study. The PR−ME extract of *P. rubra* red flower had showed the beneficial result with the highest inhibition activity of XO enzyme *in vitro* assay.

Measurement of uric acid levels in *in vivo* study was determined using uric acid standard kit assay. The concentration of uric acid is determined by a coupled of enzyme reaction which give colorimetric result within 570 nm which proportional to the

uric acid present in standard. Figure 4.13 showed the linear regression of uric acid standard curved. Calculation of uric acid in serum was made according to formula provided by standard kit assay and expressed as mg/dl.
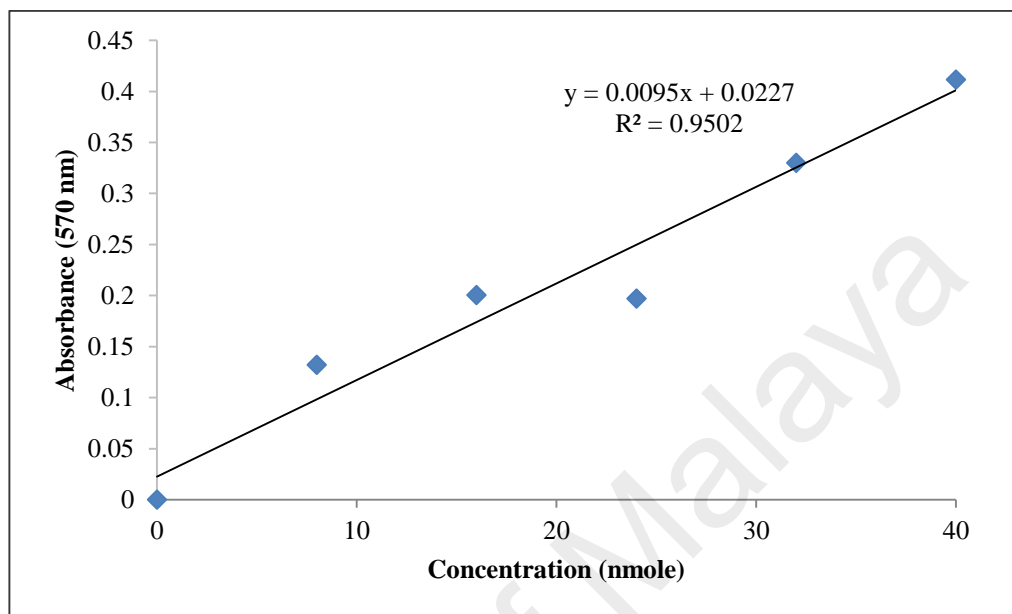
Figure 4.13. Standard curve of uric acid

Effects of PR – ME extract from *P.rubra* flowers and standard drug, allopurinol on uric acid levels in serum of hyperuricemic and normal rats are shown in Figure 4.14. As depicted from Figure 4.14, PR–ME extract from *P.rubra* flower have shown dose–dependent anti–hyperuricemic effects on serum urate levels in the normal and hyperuricemic mice were determined after 7 day oral administration. Potassium oxonate (PO) act as an uricase inhibitor treatment caused hyperuricemia in rats, as indicated by drastic increases in serum uric acid levels and this increase was prolonged to 2 hour after the injection. The serum uric acid levels of PO–hyperuricemic induced group (hyperuricemic gout-control group) were elevated significantly ($p<0.05$) to 13.65 ± 0.83 mg/dl compared to all group of control and treatment of induced hyperucemic rats at the end of the experiment.

As shown in Figure 4.14, no statistical significant difference was observed in serum urate levels between groups in normal rats non–induced with PO neither treated with test extract (baseline) which the concentration of uric acid was 3.96 ± 0.84 mg/dl with the high dose and low dose of PR–ME extract of *P. rubra* flower control group which administered with 400 mg/kg and 200 mg/kg which the concentration of uric acid was 3.82 ± 0.65 and 3.93 ± 0.43 mg/dl, respectively, without PO–induced, with as well as with 0.5 % CMC vehicle group and 3.97 ± 0.87 mg/dl, respectively. In the hyperuricemic rats, after administration with PR–ME extract of *P. rubra* flower at doses of 200 and 400 mg/kg, serum and urate levels were significantly decreased in a dose–dependent manner, compared to the gout control group. Likewise, the serum uric acid levels of hyperuricemic rats treated with high dose of 400 mg/kg PR–ME extract from *P. rubra* flower were lowered significantly by 4.44 ± 0.44 mg/dl whereas low dose of 200 mg/kg also lowered significantly by 9.71 ± 0.44 mg/dl ($p<0.05$) compared to hyperuricemic gout control group. It can be seen that dose dependent effects of PR–ME extract of *P. rubra* flower was detected in serum urate levels in rats from group of hyperuricemic induced PO group rather than control group (normal, CMC vehicle, high dose control, low dose control). In comparison to group of standard drug,allopurinol, which known as XO inhibitor has markedly reduced the level of serum urate of hyperuricemic rats to values even lower than that found in normal animals and also group from gout control rats with the concentration of serum urate level was 2.98 ± 0.589 mg/dl and 13.65 ± 0.83 mg/dl, respectively.
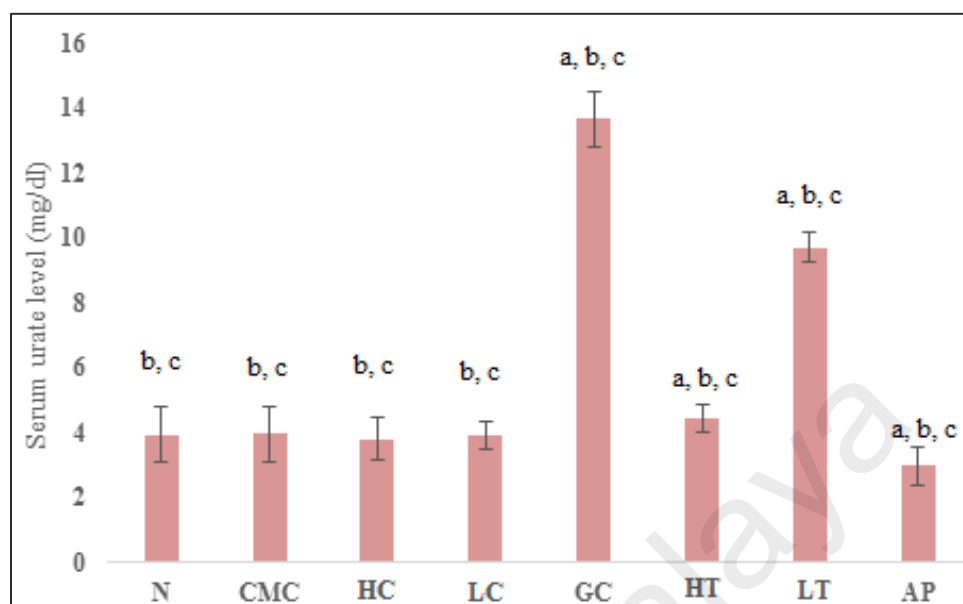
Figure 4.14 Effect of the PR–ME extract of *P.rubra* red flower on serum urate level in PO–induced hyperuricaemia in rats and control rats at 7 days. N: Rats without treated with PO, neither treated with *P. rubra* extract, CMC: Vehicle group of rats, HC: Rats administered with high dose, 400 mg/kg *P.rubra* extract, LC: Rats administered with low dose, 200 mg/kg *P. rubra* extract, GC: Gout control induced with PO, HT: Hyperuricemic rat dosed with high dose 400 mg/kg *P. rubra* extract, LT: Hyperuricemic rat dosed with low dose, 200 mg/kg *P. rubra* extract, AP: Hyperuricemic rat dosed with 10mg/kg allopurinol. The data are representative of 6 animals and expressed as mean± S.E. with a p<0.05 significant when compared to normal (N) group, b p < 0.05 significant when compared to hyperuricemic gout control (GC) group, c p<0.05 significant when compared to allopurinol (AP) group.

4.7.2 Effect of PR-ME extract on XO Activity in *in Vivo* Model

The purpose of this study is to determine the effect of PR–ME extract from *P. rubra* red flower against the activity of XO enzyme in experimental rat model *in vivo* study which including serum and also in liver . The PR–ME extract of *P. rubra* red flower had showed the beneficial result with the highest inhibition activity of XO enzyme in *in vitro* assay.

Measurement of XO activity in vivo study was determined using XO activity standard kit assay. The XO activity is determined by a coupled of enzyme reaction which give colorimetric result within 570 nm and proportional to the hydrogen peroxide generated. One unit 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. Figure 4.15 showed the linear regression of standard curved, of hydrogen peroxide standard provided by kit assay which gave the r^2 value of 0.9755 and standard curve equation of y= 0.2079x + 0.0783, where y is absorbance at 570 nm and x is concentration of standard hydrogen peroxide in nmole. Calculation of XO activity in serum and liver was made according to formula provided by standard kit assay and expressed as miliunit (mU)/mL.
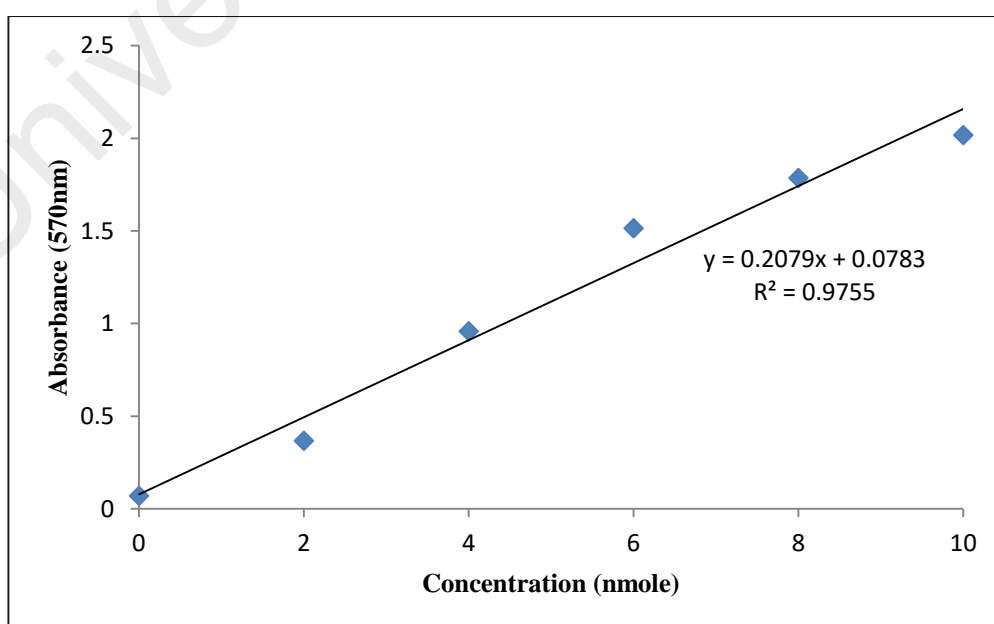
Figure 4.15 Standard Curve of hydrogen peroxide from XO standard kit assay

c. XO Inhibitory Activity in serum

The levels of uric acid are depend to a XO−catalyzed reaction. Thus, the activity of XO in serum were measured, since changes in XO activity might alter serum uric acid levels. The effect of PR−ME extract of *P. rubra* flower in *in vivo* on serum with the other group of study were illustrate in Figure 4.16 whereas the percentage of XO enzyme was shown in Table 4.24.
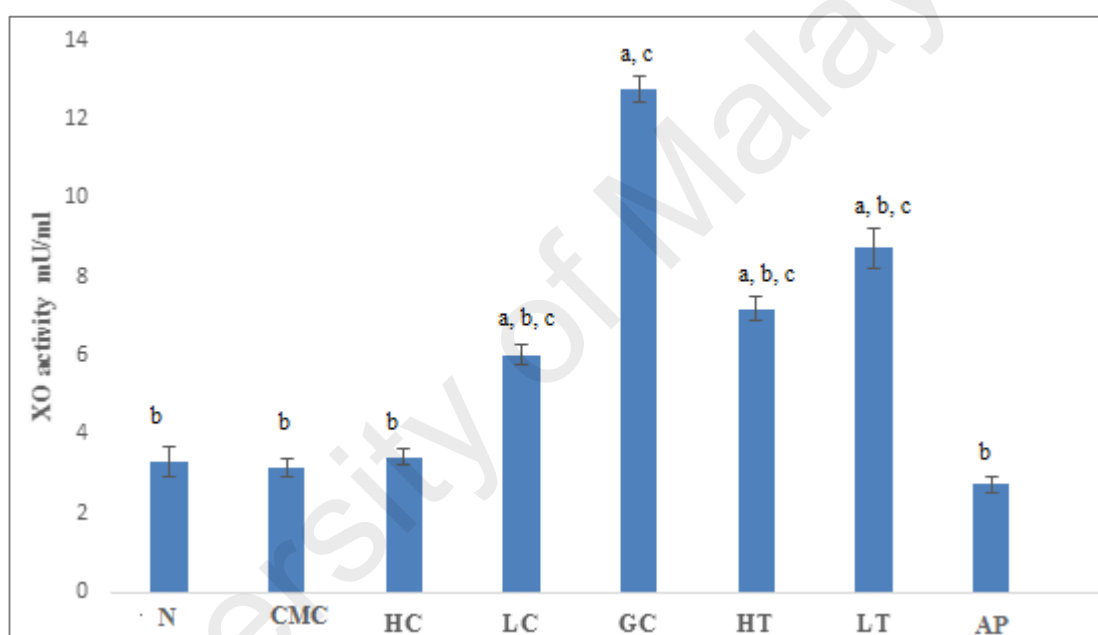
Figure 4.16 Effect of the PR−ME extract of *P. rubra* red flower on XO activity in serum of PO−induced hyperuricaemia in rat and normal control rat at 7 days. N: Rats without induced with PO neither treated with *P. rubra* extract and standard drug allopurinol, CMC: Vehicle group of rats, HC: Rats administered with high dose, 400 mg/kg *P. rubra* extract, LC: Rats administered with low dose, 200 mg/kg *P. rubra* extract, GC: Hyperuricemic gout control induced with PO (280 mg/kg), HT: Hyperuricemic rat dosed with high dose 400mg/kg *P. rubra* extract, LT: Hyperuricemic rat dosed with low dose, 200mg/kg *P.rubra* extract, AP: Hyperuricemic rat dosed with 10mg/kg Allopurinol. The data are representative of 6 animals and expressed as mean± S.E. with a $p<0.05$ significant when compared to normal (N) group, b $p < 0.05$ significant when compared to hyperuricemic gout control (GC) group c $p<0.05$ significant when compared to allopurinol (AP) group.

As shown in Figure 4.16, dose dependent effects of high dose, 400 mg/kg and low dose, 200 mg/kg of PR−ME extract from *P.rubra* flowers in serum can be seen only PO – induced hyperuricemic rats. There were no statistically significant difference in serum XO activity ($p<0.05$) between group of normal untreated extract and non-induced of PO (baseline) which the XO activity was 3.29 ± 0.38 mU/mL, 0.5 % CMC vehicle group which the XO activity was 3.15 ± 0.24 mU/mL, high control group (dose 400 mg/kg) which the XO activity was 3.42 ± 0.2 mU/mL and positive control group, allopurinol which the XO activity was 2.74 ± 0.21 mU/mL Nevertheless, XO activity in low control group (200 mg/kg) was significantly increased to 6.01 ± 0.26 mU/mL compared to normal (baseline) group ($p<0.05$). Refer to Table 4.22, administration of high dose, 400 mg/kg of PR−ME extract of *P. rubra* flower among non−induced of PO rats was able to cause weak inhibition of serum which was 3.95 %. Instead, low dose, 200 mg/kg of PR−ME extract of *P. rubra* flower not capable to inhibited the XO and in fact, increased the XO activity more than normal value.

It was noted that PO successfully play a role as competitive uricase inhibitor since the XO activity in hyperuricemic gout control group (GC) was significantly increased ($p<0.05$) and was the highest among the other seven groups of studies (12.77 ± 0.33 mU/mL). In hyperuricemic rats, after administration with PR−ME extract of red flowers at doses of 200 and 400 mg/kg, XO activity were significantly decreased in a dose – dependent manner, compared to the gout control group. As represented in Figure 4.16, the XO activity of hyperuricemic rats treated with high dose of 400 mg/kg PR−ME extract from *P. rubra* flower were lowered significantly by 7.18 ± 0.30 mU/mL with the percentage of XO inhibition was 43.77 % whereas low dose of 200 mg/kg also lowered significantly by 8.73 ± 0.52 mU/mL ($p<0.05$) and showed percentage of XO inhibition was 31.64 % compared to gout control group. Interestingly, there was no statistical significant difference between the group hyperuricemic PO−induced which

treated with high dose, 400 mg/kg PR–ME extract of *P. rubra* flower with the group of normal rats that only given low dose, 200 mg/kg PR–ME extract of *P. rubra* flower. However, standard drug, allopurinol which acted as positive control in this study exhibited the best inhibition of XO activity (Table 4.24) from those two different dose of PR–ME extract of *P. rubra* flower which the XO activity of rats treated with allopurinol was nearly reached and no statistical different with the normal (baseline) rats.

d. XO Inhibitory Activity in liver

The levels of uric acid are depend to a XO–catalyzed reaction. Thus, the activity of XO in liver were measured, since changes in XO activity might alter serum uric acid levels. In fact, XO activity is present in significant level in liver. The effect of PR–ME extract of *P. rubra* flower in *in vivo* model on liver with the other group of study were illustrate in Figure 4.17 whereas the percentage of XO enzyme was shown in Table 4.24.
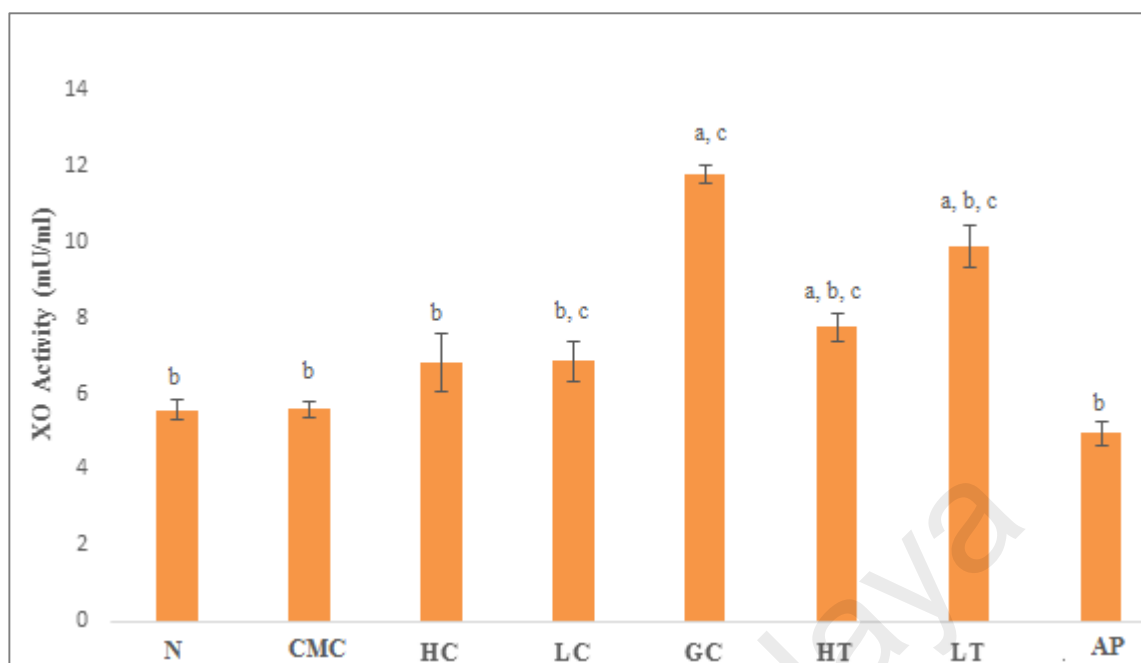
Figure 4.17 Effect of the PR–ME extracts of *P. rubra* flower on XO activity in liver PO–induced hyperuricaemia in rat and normal control rat at 7 days. N: Rats without treated with PO, neither treated with *P. rubra* extract, CMC: Vehicle group of rats, HC: Rats administered with high dose, 400 mg/kg *P. rubra* extract, LC: Rats administered with low dose, 200 mg/kg *P. rubra* extract, GC: Hyperuricemic gout control induced with PO, HT: Hyperuricemic rat dosed with high dose 400 mg/kg *P. rubra* extract, LT: Hyperuricemic rat dosed with low dose, 200 mg/kg *P. rubra* extract, AP: Hyperuricemic rat dosed with 10mg/kg allopurinol. The data are representative of 6 animals and expressed as mean± S.E with a $p<0.05$ significant when compared to normal (N) group, b $p< 0.05$ significant when compared to hyperuricemic gout control (GC) group, c $p<0.05$ significant when compared to allopurinol (AP) group.

Figure 4.17 shows the dose dependent effects of both doses of PR–ME extract of *P. rubra* flower, 400 mg/kg and 200 mg/kg only can be seen on XO activity in liver from PO–induced hyperuricemic rats but not in normal rats after 7 days of oral administration. There were no statistically significant difference in liver XO activity ($p<0.05$) between group of normal untreated extract and non–induced of PO (baseline) which the XO activity was 5.58 ± 0.249 mU/mL with the other four groups including 0.5 % CMC vehicle group which the XO activity was 5.59 ± 0.224 mU/mL, high control group (dose 400 mg/kg) which the XO activity was 6.83 ± 0.761 mU/mL, low control group (200 mg/kg) give the XO activity as 6.85 ± 0.530 mU/mL and positive control

group, allopurinol which the XO activity was 4.96 ± 0.312 mU/mL when compared to normal without induced of PO and treatment of extract. Moreover, it was found that no statistical significant difference ($p<0.05$) between the high control group (dose 400 mg/kg) with the low control group (dose 200 mg/kg) among non–induced PO rats. Therefore, the administration of PR–ME extract of *P. rubra* flower does not give any effect to XO activity of normal rats as these doses did not produced any inhibition of XO activity in liver (Table 4.24). Furthermore, PO played its role as uricase inhibitor as the gout control group exhibited the significantly increased the XO activity in liver compared which was 11.77 ± 0.232 mU/mL to other group of study but slightly less than XO activity in serum of the same group (12.77 ± 0.331 mU/mL). On the other hand, Figure 4.17 also demonstrated that XO activity in liver among hyperuricemic rats was significantly decreased in a dose–dependent manner with oral administration of these two doses (200 and 400 mg/kg) of PR–ME extract from *P. rubra* flower after 7 day duration in *in vivo* study when compared to the gout control group. As shown in Figure 4.17 and also in Table 4.24, the XO activity of hyperuricemic rats treated with high dose of 400 mg/kg PR–ME extract from *P. rubra* flower were lowered significantly by 7.76 ± 0.381 mU/Ml with percentage of XO inhibition was 34.07 % whereas low dose of 200 mg/kg also lowered significantly by 9.88 ± 0.536 mU/mL ($p<0.05$) and showed percentage of XO inhibition was 16.06 % when compared to gout control group. Nevertheless, standard drug, allopurinol which acted as positive control in this study was assumed as most potent XO inhibitor since the XO activity of liver was reduced significantly ($p<0.05$) to 4.96 ± 0.312 mU/mL when compared to gout control group, in spite of the percentage of XO inhibition activity of was lower (57.86 %) rather than in serum of the same group of rats (78.54 %).

Table 4.24. XO Activity and Percentage of XO inhibition of serum and liver from normal and hyperuricemic induced groups of rats after oral administration of PR–ME extract of *P.rubra* flowers within 7 days. The data are representative of 6 animals and expressed as mean ± S.E. with a p<0.05 significant when compared to normal (N) group, b p < 0.05 significant when compared to gout control (GC) group, c p<0.05 significant when compared to allopurinol (AP) group.

Group of Treatment	XO Activity (mU/mL)		XO Inhibition (%)	
	Serum	Liver	Serum	Liver
Normal rats (Baseline)	3.29 ± 0.38 b	5.58 ± 0.25 b	-	-
Vehicle (CMC 0.5 %)	3.15 ± 0.24 b	5.59 ± 0.22 b	-	-
High control (400 mg/kg)	3.42 ± 0.20 b	6.83 ±0.76 b	3.95	-
Low control (200 mg/kg)	6.01 ± 0.26 a, b, c	6.85± 0.53 b, c	-	-
Hyperuricemic Group Control	12.77 ± 0.33 a, c	11.77± 0.23 a, c	-	-
High treatment (400 mg/kg)	7.18 ± 0.30 a, b, c	7.76± 0.38 a, b, c	43.77	34.07
Low treatment (200 mg/kg)	8.73 ± 0.52 a, b, c	9.88± 0.54 a, b, c	31.64	16.06
Allopurinol treatment	2.74 ± 0.21 b	4.96 ±0.31 b	78.54	57.86

4.8 The Effect of PR-ME Extract on Body Weight

The effect of continuously consuming *P. rubra* red flower on health was examined by measuring body weights day 0, day 1, day 3, day 5 and day 7 as depicted in Table 4.25. Figure 4.18 showed the changes on body weight between normal and treated groups. The body weights of all the rats gradually increased throughout the experiment, regardless of their treatment group. Since there were no significant differences of body weight between the treatments groups, therefore, body weight of all rats was not influenced by extract of *P. rubra* flower. This indicated that the *P. rubra* flower extract is not toxic to the rats.

The data of changes in body weight were analyzed by one way ANOVA followed by Duncan and Dunnett's post hoc multiple comparison test using SPSS 22.0 software version. The values were considered statistical significant when $p < 0.05$ level. All the results were expressed as mean ±S.E.

Table 4.25. Mean Body Weight of PR–ME extract from *P. rubra* red flower on control groups and treated groups of animal. The data are representative of 6 animals and expressed as mean ± S.E.

Animal Group	Mean Body weight (g)				
	Day 0	Day 1	Day 3	Day 5	Day 7
Normal (Baseline)	212.30 ±0.23	213.40 ±0.42	215.10 ±0.46	216.90 ±0.42	218.10 ±0.32
Vehicle 0.5 % CMC	212.70 ± 0.62	213.80 ± 0.65	215 ± 0.68	216.50 ±0.43	218 ± 0.41
High Control (400 mg/kg)	212.20 ± 0.48	213.90 ± 0.62	215.20 ±0.70	216.80 ±0.83	218.20 ±0.83
Low Control (200 mg/Kg)	212.70 ± 0.83	213.80 ±1.14	215.20 ±1.39	216.50 ±1.92	217.60 ±2.03
Hyperuricemic Control (PO)	212.50 ± 0.62	214.20 ±0.61	215.20 ±0.42	216.30 ±0.41	217.60 ±0.39
High Treatment (PO+HPR) (400 mg/kg)	212.80 ±1.35	214.50 ±1.57	216.10 ±1.63	216.90 ±1.65	218.20 ±1.68
Low Treatment (PO+LPR) (200 mg/kg)	212.70 ±0.79	213.90 ±0.87	215.40 ± 0.94	216.90 ±0.88	218.40 ±1.03
Standard Allopurinol (PO+Allo)	212.30 ± 0.98	213.50 ± 1.06	215.20 ± 1.08	216.80 ±1.01	218.20 ± 1.10
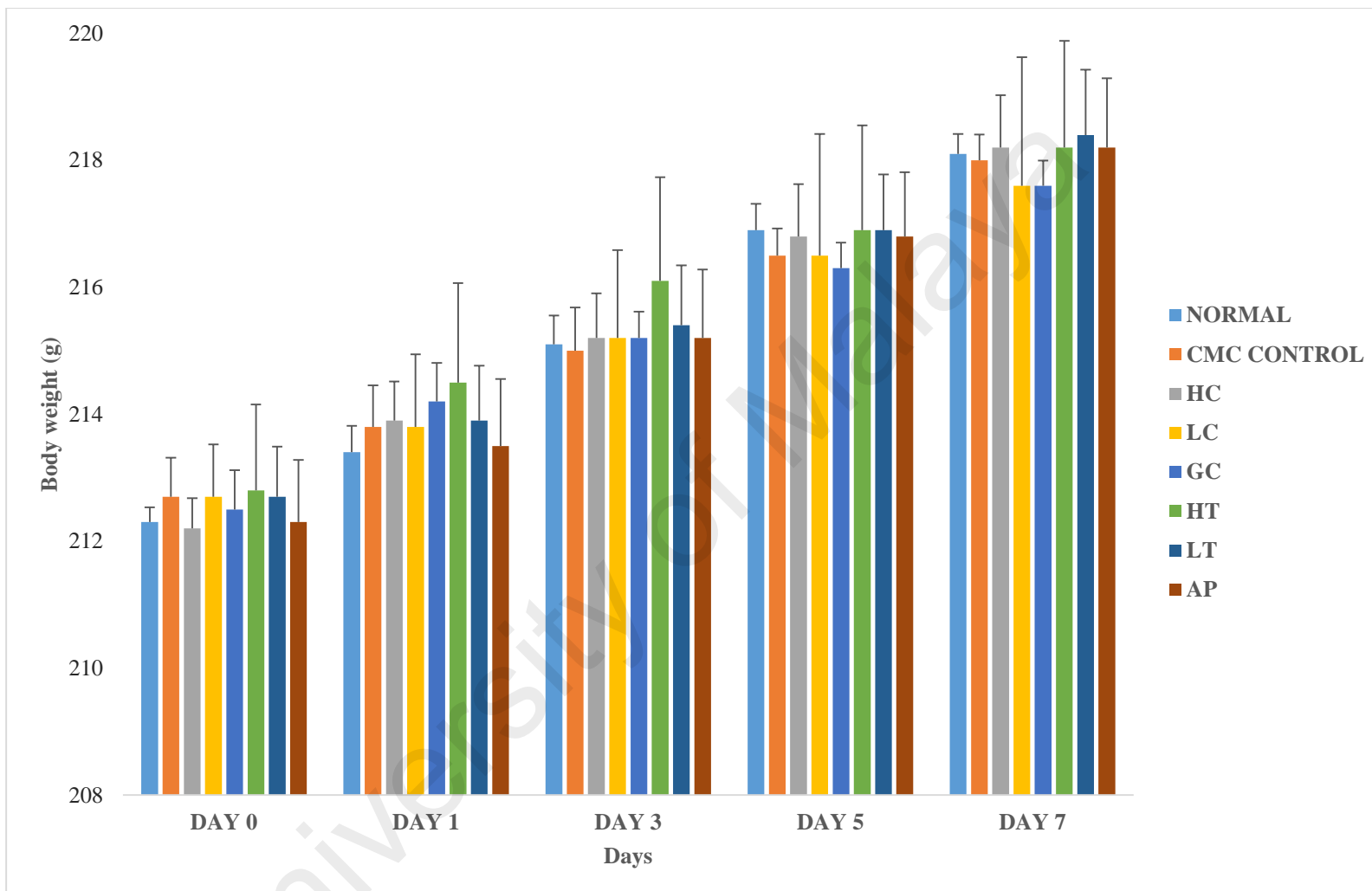
Figure 4.18. Effect of PR-ME extract of *P. rubra* red flower on body weight of rats. N: Rats without treated with PO, *P. rubra* extract, CMC Control: Vehicle group of rats, 0.5 % CMC, HC: Rats administered with high dose, 400 mg/kg *P. rubra* extract, LC: Rats administered with low dose, 200 mg/kg *P. rubra* extract, GC: Gout control induced with PO, HT: Hyperuricemic rat dosed with high dose 400 mg/kg *P. rubra* extract, LT: Hyperuricemic rat dosed with low dose, 200 mg/kg *P. rubra* extract, AP: Hyperuricemic rat dosed with 10 mg/kg allopurinol. The data are representative of 6 rats and expressed as mean± S.E.

CHAPTER 5

DISCUSSIONS

5.1 Thin Layer Chromatography (TLC) Profile of *Plumeria rubra* Flower Extracts

The thin layer chromatography profile of extracts from red flowers *Plumeria rubra* showed the presence of essential oils, phenols, flavonoids and terpenoids. The chemical reagents were carried out in order to detected the phytochemical compounds existed in the red flower *P.rubra*. The formation of blue spots with folin-ciocalteu reagent indicated the presence of phenols in the extracts of *P.rubra* flower. Besides, the presence of terpenoids was carried out using vanillin–sulphuric acid reagent with the formation of purple spots while the formation of red spots or other colour indicated the presence of phenols and flavonoids. Furthermore, anisaldehyde–sulphuric acid was another chemical reagent which detected the presence of essential oils with the formation of blue spot, whereas the flavonoid compound was detected with the formation of red spots. This finding was agreed with the previous research by Zaheer *et al.* (2010) which confirmed the presence of flavonoid compound in methanol extract of *P.rubra* flower through preliminary phytochemical screening.

Chloroform and methanol are both polar solvent which used to extract polar compound of lipids, phenolic compound (Hossain *et al.* 2011), and flavonoids (Bimakr *et al.* 2011). The higher polarity of methanol solvent compared to chloroform solvent showed the presence of terpenoids in methanol extract of thin layer chromatography profile (Iwalewa *et al.* 2003). Moreover, the best method for extraction of phenolic compounds was solvent extraction with water or methanol (Goli *et al.* 2005). On the other hands, hexane extract contain fewer compounds as compared to chloroform,

methanol and water extracts. The reason may be due to solvent hexane was non-polar solvent which not many chemical constituents could be extracted from this solvent resulted to slightly amount of phenols was detected through folin and vanilin reagent. Beside this, the observation of essential oil of hexane extract from flower of *P. rubra* is in agreement with research analyzed by Tohar *et al.* (2006) that the flower of genus *Plumeria* contain various of volatile component which benefit to fragrance and medicine industry. In addition, mostly lipids can be found in nonpolar phase of solvent system, and proteins, amino acids, and other hydrophilic molecules such as phenols and flavonoid remained in polar phase (Wanasundara & Shahidi, 1994).

From the result, the TLC profile indicated that no alkaloids within the flowers of *P.rubra*. This result could be explained that alkaloids tend to accumulate in very active tissues such roots, leaves, vascular sheath and latex (Robinson, 2012).

5.2 Liquid Chromatography Mass Spectrometry (LCMS/MS) Analysis of *P. rubra* Flower Extracts

The separations of the phytochemical compounds in extracts from *P. rubra* red flowers were carried out using Liquid Chromatography Mass Spectrometry tandem with mass spectrometry (LCMS/MS) in order to determine the phytochemical presence in flowers of *P. rubra*. Analysis with LCMS/MS in PR–ME (methanol) extract from *P.rubra* flowers showed the presence of 3–caffeyolquinic acid , 5–caffeoquinic acid, chlorogenic acid, citric acid, kaempferol–3–O–glucoside, kaempferol–3–rutinoside, kaempferol, quercetin 3–O–α–L–arabinopyranoside , quercetin , quinic acid and rutin. Analysis with LCMS/MS in PR-WE (water) extract from *P.rubra* flowers showed the presence 1,3- Dicaffeoquinic acid , 3,3–di–O–methylellagic acid, 3–Caffeyolquinic acid, kaempferol–3–O–glucoside, kaempferol–3–rutinoside, quercetin and quinic acid. It should

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be noted that the phytochemical compounds which detected by LCMS/MS method in present study are difference compared from the phytochemical compounds that have been identified in flowers *P.rubra* as reported in previous study. Contrary to present study, phytochemical compounds including of myristic acid, linoleic acid, lauric acid and palmitic acid (Tohar *et al.* 2006), plumericidine (Ye *et al.* 2009), two anthocyanins which are cyanidine $3-O-\beta-(2''-$glucopyranosyl$-O-\beta-$galactopyranoside) and $3-O-\beta-$galactopyranoside (Byamukama *et al.* 2011), rubranoside (=7$-O-\alpha-L-$rhamnopyranosyl$-$4'$-O-\beta-D-$glucopyranosylnaringenin), rubrajaleelol, rubrajaleelic acid,1$-\alpha-$plumieride and plumieride $-p-z-$coumarate (Akhtar *et al.* 2013) were identified in red flowers of *P.rubra*. Nevertheless, these compounds was unable to be detected in the present study could be due to the low amount of compound presence and also different approach of extraction process in these research.

5.3 Total Phenolic and Flavonoid Content of *P .rubra* Flower Extract

The total phenolic and flavonoids compounds were quantified since the phenolic and flavonoids has been qualitatively detected in extracts of *P. rubra* red flowers. Methanol extract (PR$-$ME) showed the highest contents of both TPC and TFC followed by water extract (PR$-$WE), chloroform extract (PR$-$CE) and hexane extract (PR$-$HE). The high TPC in PR$-$ME of red flowers *P.rubra* flowers could be due to the presence of phenolic compounds which are $3-$Caffeyolquinic acid, $5-$Caffeoquinic acid, Chlorogenic acid and quinic acid. The total flavonoid contents in flowers from *P. rubra* was also highest in PR$-$ME and the presence of kaempferol$-3-O-$glucoside, kaempferol$-3-$rutinoside, Kaempferol, Quercetin $3-O-\alpha-L-$arabinopyranoside , quercetin and rutin in methanol extract of *P. rubra* flower might be contributing for high contents of

flavonoids. The result of TPC and TFC of flowers from *P.rubra* were varied among the four extracts.

Variation of result from different extract of *P. rubra* flowers could be due to the different amount of non-phenolic compound which was flavonoid has capable to formed complex with ion aluminium thus gave pink color reaction. In addition, methanol extract yielded highest phenolic and flavonoid content which are in agreement with Yao *et al.* (2004), reported that methanol is the best solvent in the extraction of phenolic compounds due to its ability to inhibit the reaction of polyphenol oxidase that causes the oxidation of phenolic and its ease of evaporation compared with water. Instead, the total phenolic contents and total flavonoid contents of PR–HE extract were much lower, which are in agreement with Sahreen *et al.* (2010) and Medini *et al.* (2014) as both of these finding indicated the low content of phenols and flavonoids from the hexane extract. Beside this, the results also suggest that the extractability of polyphenols is influenced by the polarity and viscosity of the solvents used (Turkmen *et al.* 2006 and Hayouni *et al.* 2007). This results are agreed the finding by Barreira *et al.* (2008) which revealed that *P. rubra* flower contained remarkable levels of phenols and flavonoids and the presence of these compounds are according to the polarity of solvent used.

5.4 Antioxidant Activity Assays of *P.rubra* Flower Extracts

The antioxidant activities of red flowers from *P. rubra* extracts were evaluated with 2,2–diphenyl–1–picrylhydrazyl (DPPH) radical scavenging assay, Ferric Reducing Power Assay (FRAP), Metal Chelating Assay, Hydrogen peroxide radical scavenging assay, Nitric Oxide radical scavenging assay and Superoxide radical scavenging assay. On the other hand, the present study showed that the methanol extract of red flower from *P.rubra* comprised of 3–caffeyolquinic acid , 5–caffeoquinic acid,

chlorogenic acid, kaempferol−3−O−glucoside, kaempferol−3−rutinoside, kaempferol, quercetin 3−O−α−L−arabinopyranoside , quercetin , quinic acid and rutin as detected by LCMS/MS analysis. These compounds are might attributed to all of six antioxidant assays in the current study and the antioxidant activity of these compounds from red flower of *P.rubra* were not reported yet in previous research. Besides, the result of all these antioxidant activities especially in PR−ME might be due to synergistic effect of all detected compounds and several compounds contain in methanol extract had been reported from previous literatures. On the other hand, the types of chlorogenic acids presence in PR−ME extract with different number of phenolic –OH would also affect the antioxidative activity. As of now, many literatures reported the antioxidant activity of 3−O−caffeoylquinic acid rather than 5−O−caffeoylquinic acid as both of them might contributed to antioxidant activity with different mechanism.

Some of the data of the antioxidant activity assays indicated the small range of SE. The reason is the present study had chose SE rather than SD in order to describe variation as SE gives the smallest of the error bars. In fairness, SEM is a perfectly legitimate descriptor of variation in research.

5.4.1 DPPH (2, 2-diphenyl-1-picrylhydrazyl) Radical Scavenging Activity of *P.rubra* Flower Extracts

The DPPH scavenging activity was carried out to evaluate the ability of antioxidant to scavenge free radicals. DPPH is considered to be a model of a stable lipophilic radical. A chain reaction of lipophilic radicals is initiated by lipid auto-oxidation. Antioxidants react with DPPH, reducing the number of DPPH free radicals to the number of their available hydroxyl groups (Khaliq *et al.* 2015). The more antioxidant compound in the extract, the more DPPH reduction will occur. The degree of

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discoloration from violet colour of DPPH radical to pale yellow colour and adsorption intensity also decreased according to the number of electron captured and indicates the scavenging potentials of the antioxidant extract (Bastos *et al.* 2007). The higher intensity of DPPH reduction is related to high scavenging activity performed by particular compounds. In the present study, the scavenging activity of *P. rubra* flowers extracts were compared with ascorbic acid which is well known natural antioxidants. In general, the scavenging activity of *P. rubra* flower extracts was increased with increasing concentration. In this assay, the IC_{50} of standard ascorbic acid was 29.6 μg/mL. The weaker pattern of scavenging activity was observed for the PR–CE compared to PR–ME. PR–HE was generally least reactive with lowest percentage of DPPH inhibition. The scavenging activities of PR–HE was suited in most research that hexane extract showed hyperbolic curve. This observation is fairly common due to not many non – polar compounds could act as potent antioxidant (Kulkarni *et al.* 2004).

The highest inhibition of DPPH radical in PR–ME is due to the presence of flavonoids compound which are quercetin, kaempferol and rutin as well as phenolic acid which are 3–O–Caffeyolquinic acid, 5–O–Caffeoquinic acid, Chlorogenic acid and quinic acid that has been detected in LCMS/MS. Both the number and configuration of H–donating hydroxyl groups are the main structural features that influence the antioxidant capacity of these flavonoids (Pannala *et al.*, 2001). The quercetin and kaempferol structure contain of catechol arrangement which prerequisite to reinforce DPPH scavenging activity. Nevertheless, according to Wang *et al.* (2012), the glycosylation of flavonol as the detected phytochemical compounds in present study, kaempferol – 3 – glycoside, kaempferol–3–rutinoside and quercetin 3–O–α–L–arabinopyranoside on both OCH3 and OH groups reduced the DPPH free radical scavenging potential. The result of PR-ME of the present study also show agreement with Yang *et al.* (2008) and Lue *et al.*

(2010) which extracts contain of rutin has capability to scavenge DPPH radicals. Moreover, the presence of ortho–dihydroxyl group in the B–ring of rutin as well as the sugars at the 3 position makes it strong free radical scavenging activity (Sintayehu, 2012). On the other hand, quinic acid presence in both PR–ME and PR–WE could act as ortho- or para diphenolic group was also effective in free radical scavenging by electron transfer (Xu *et al.* 2008). In addition, the other study by Yuan *et al.* (2012) stated that the caffeoyl group in 3–O–caffeolquinic acid is the key active site exhibiting antiradical activities in the separated phenolic compounds. The radical scavenging activities of tested phenolic compounds are slightly affected by the position of the caffeoyl group on quinic acid. Thus, the phenolic compounds including of 3–O–Caffeoylquinic acid, 5–O–caffeoylquinic acid, chlorogenic acid and quinic acid might play a role in order to scavenge DPPH radical in present study.

5.4.2 Ferric Reducing Antioxidant Power (FRAP) assay of *P.rubra* Flower Extracts

The Ferric Reducing Antioxidant Power (FRAP) assay was carried out to evaluate the ability of extracts from *P. rubra* red flowers to reduce the ferum ion in relation to its antioxidant activity. The ferric reducing activity of all extracts of *P.rubra* flower were analyzed based on the reduction of ferric–TPTZ (2, 4, 6– tri(2–pyridyl)–1,3,5–triazine) to blue ferrous TPTZ. Antioxidant compounds which act as reducing agent exert their effect by donating hydrogen atom to ferric complex and thus break the radical chain reaction. The higher the absorbance is, the higher is the antioxidant activity which is indicated by the high FRAP value (Ghafar *et al.* 2010). The highest FRAP value was displayed from PR – ME while the moderate polarity of solvent which was PR – CE demonstrated the moderate reducing power and lowest FRAP value was displayed from PR–HE. It was observed that PR–ME and PR–WE were able to donate electron, hence they should be able to donate electrons to free radicals in actual biological or food

systems, making the radicals stable and unreactive. The results obtained may have partly been contributed by the phenolics and flavonoids as detected in LCMS/MS in PR–ME and PR–WE such as 3–O–caffeyolquinic acid, 5–O–caffeoquinic acid, 1, 3–dicaffeoquinic acid, chlorogenic acid, 3, 3–di–O–methylellagic acid, kaempferol–3–O–glucoside, kaempferol–3–rutinoside, kaempferol, quercetin 3–O–arabinosyl glucoside, quercetin, quinic acid and rutin. Pu *et al.* (2015) stated that both quercetin and kaempferol are flavonoids which responsible to give high of FRAP value due to the number and position of hydroxyl groups and the different flavonoid skeletons. The other phytochemical compounds that reported to be acted as reducing agent of ferric–TPTZ (2, 4, 6–tri(2–pyridyl)–1,3,5–triazine) are rutin (Firuzi *et al.* 2005) as well as chlorogenic acid, 3–O–caffeoylquinic acid and 5–O–caffeoylquinic acid (Shi *et al.* 2009).

5.4.3 Metal Chelating Activity of *P.rubra* Flower Extract

In the metal chelating assay of red flowers of *P. rubra* extracts showed that PR–ME produced the highest metal chelating activity. The present result was an agreement with Rohman *et al.* (2010) as the higher metal chelating activity was indicated by the smaller IC_{50} value. Ion Ferrous (Fe^{2+}) is an unstable form of iron which contributed to formation of ROS that cause lipid peroxidation, nucleic acid or protein damage whereas Ferric (Fe^{3+}) is an inactive but more stable ion (Chvatalova *et al.* 2008). The complex formation of ferrozine with Fe^{2+} can be disturbed by chelating agents via inhibition of heavy metal. According to Gordon, (1990), the effective chelating agents able to form σ-bonds with a metal as secondary antioxidants due to their capability to reduce the redox potential thus stabilizing the oxidized form of the metal ion.

The highest of metal chelation in PR–ME might be contributed to phytochemical compounds that has been analyzed through LCMS/MS which are 3–O–caffeoylquinic acid, 5–O–caffeoylquinic acid, chlorogenic acid, quinic acid and quercetin.

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Polyphenols compound has been known to be attributed to antioxidant activity of plant by scavenging free radicals, iron chelating mechanism and changed the activity of enzymes by inhibiting oxidases (Melidou *et al.* 2005; Chvatalova *et al.* 2008). In addition, phenolic compounds interfered the oxidation of lipids and other molecules through rapid donation of a hydrogen atom to free radicals which this reaction leading to formation of stable substance phenoxy radical intermediates also act as terminators of propagation route by reacting with other free radicals (Dai & Mumper, 2010). Moreover, phenolic compounds such as 3−O−caffeoylquinic acid and 5−O−caffeoylquinic acid posses ideal structure chemistry for free radical scavenging activities because they have phenolic hydroxyl groups that are prone to donate a hydrogen atom or an electron to a free radical and also extended conjugated aromatic system to delocalize an unpaired electron. According to Luzia *et al.* (1998), 5−O−caffeoylquinic acid could acts as metal chelator due to presence orthohydroxy in its chemical structure. In addition, chlorogenic acid revealed good metal chelating activity due to more complex structure of this phenolic acid which is a caffeic acid ester linked to quinic acid (Apati *et al.* 2003; Andjelkovic *et al.* 2006). The other phenolic compound, quinic acid also showed strong ability to chelate metal ions involved in the production of free radicals (Seabra *et al.* 2006). The fewer the number OH groups, the lower probability of hydrogen loss posed and the lower the probability of oxidation of the flavonoid and the reduction of the metal (Fernandez *et al.* 2002). On the other hand, quercetin was known as iron chelating and iron stabilizing properties. The chemical structure of quercetin which contain catechol moiety in the B ring has been participated in metal chelation (Sestili *et al.* 1998; Prochazkova *et al.* 2011).

5.4.4 Hydrogen peroxide Radical Scavenging Activity of *P. rubra* Flowers Extracts

Hydrogen peroxide radical scavenging assay was carried out to estimate the ability of extracts from red flower of *P.rubra* to scavenge free radical, hydrogen peroxide. Hydrogen peroxide can be formed in *in vivo* by various oxidizing enzymes such as superoxide dismutase. It can permeate through biological membranes slowly oxidizing number of compounds. Hydrogen peroxide is used in the respiratory burst of activated phagocytes (Mac Donald *et al.* 2006). Although hydrogen peroxide itself is not very reactive (Namiki, 1990), together with the superoxide radical anion, can damage many cellular components thus, can be converted into other ROS, including hydroxyl radicals which is found to be main reason for toxicity associated with hydrogen peroxide (Ma *et al.* 2011). Moreover, hydrogen peroxide can deactivate few enzymes involved in cellular energy production such as glyceraldehyde – 3 – phosphate dehydrogenase found in glycolytic pathway (Hyslop *et al.* 1988) as well as aconitase and a-ketoglutarate dehydrogenase found in Krebs cycle (Tretter and Vizi, 2000) through oxidation of essential thiol (– SH) groups. Therefore, it biologically advantageous for hydrogen peroxide scavenger to control the amount of hydrogen peroxide that is allowed to accumulate.

In the hydrogen peroxide scavenging activity assay, the scavenging activity was found to increase with increasing concentration of *P.rubra* flower extracts. The present study exhibited the highest percentage of hydrogen peroxide scavenging activity in PR–ME of *P. rubra* flower. Interestingly, the other extract of *P. rubra* flower, which are PR–WE, PR–CE and PR–HE exhibited weaker pattern of hydrogen peroxide scavenging activity as their inhibition percentage of hydrogen peroxide radical did not reach 50 % even at the highest concentration tested. However, based on estimated IC_{50} value the scavenging activity of PR – ME extract was relatively lower than standard,

ascorbic acid. Therefore, the study indicates PR–ME of *P.rubra* flower has demonstrated mild scavenging activity on hydrogen peroxide. The scavenging activity of hydrogen peroxide radical in PR–ME are due to the presence of compounds which detected by LCMS/MS analysis including $3-O-$caffeoylquinic acid, kaempferol and quercetin. Scavenging of hydrogen peroxide by the plant extracts may be attributed to their phenolic contents, which donate electron to hydrogen peroxide, thus reducing it to water (Sharma *et al.* 2012). Furthermore, the other compound such as quercetin possessing all the structural requirement of a flavonol antioxidants which including $5-$hydroxyl$-4-$keto substitution in the A ring, the 2, 3 – double bond promoting conjugation between the ring and 3, 4 dihyroxy catechol substitution in the B ring which these characteristics give quercetin as good antioxidant (Apak *et al.* 2007) and anti-radical activity (Seyoum *et al.* 2006). Anti–radical activity of bioactive compound are mainly correlated by the number of –OH group preferably in ortho position in one another bonded to aromatic ring (Sroka & Cisowki, 2003). The mild scavenging activity on hydrogen peroxide radical was due to less bioactive compound which responsible to scavenge hydrogen peroxide radical. Meanwhile quercetin flavonoids also could be acted as pro-oxidant which exacerbate the oxidative stress derived from hydrogen peroxide (Prokazkova *et al.* 2011). Moreover, hydrogen peroxide is necessary to peroxidases catalyzed reactions and it scavenging could be the cause of a lower enzymatic oxidative process.

5.4.5 Nitric Oxide Radical Scavenging Activity of *P.rubra* Flower Extracts

The nitric oxide (NO) radical scavenging activity was carried out to determine the ability of extracts from *P.rubra* red flowers to scavenge nitric oxide radical. NO is generates spontaneously from sodium nitroprusside in an aqueous solution at physiological pH, interacts with oxygen to produce nitrite ions which can be estimated using a griess reagent. Scavengers of nitric oxide act against oxygen, leading to reduced production of nitrite ions which can be monitored at 546 nm. Nitric oxide (NO) radical is involved in several pathological diseases, such as chronic inflammation, aut oimmune diseases and rheumatoid arthritis, were also proposed to be closely associated with its overproduction and inappropriate expression of cytokines (Szekanecz & Koch, 2007; Kobayashi, 2010; Hong *et al.* 2015). During inflammation, the level of nitric oxide increases and an overproduction can cause damage to cell macromolecules and therefore injuries to host tissue. Besides its own toxicity, this radical can further react with other species instigating even more toxic radicals, such as peroxynitrite, which results from its reaction with superoxide. (Royer *et al.* 2011: Pereira *et al.* 2010).

The NO scavenging activity was found to increase with increasing concentration of *P. rubra* red flower extracts. The present study indicated that PR−ME and PR−WE could inhibited the nitrite formation by directly competing with oxygen in the reaction with nitric oxide. The high inhibition of red flowers PR−ME and PR−WE might be due to the presence of phenolics and flavonoids compound which including hydrocinnamic acid (3−O−caffeolquinic acid, 5−O−caffeolquinic acid), quinic acid, flavonoid (quercetin, and kaempferol). In fact, both 5−O−caffeoylquinic acid, quercetin and kaempferol have already revealed antioxidant capacity, in several systems (Han *et al.* 2004; Ferreres *et al.* 2008). Quercetin was claimed as most potent of flavonoids family as scavenger of ROS, including RNS like NO· (Vanacker *et al.* 1995; Haenen & Bast,

1999). As a result, several previous studies have shown that flavonoids which is quercetin able to inhibit NO production which in response to inflammatory stimuli. In fact, quercetin not only prevent the occurrence of oxidative stress but also help mitigate inflammation (MacNee, 2001; Rahman, 2002: Nair *et al.* 2006). Indeed, it has already been shown that quercetin and kaempferol were able to inhibit NO production to act against NOS isoforms boh by inhibition of induced NOS (iNOS) activity or down-regulation of iNOS expression from different cell types (Rangan *et al.* 1999: Chen *et al.* 2005; Hamalainen *et al.* 2007). Furthermore, quercetin also inhibits ROS/RNS generation, inducible nitric oxide synthase expression and NF–kB activation in IL–1b–activated rat hepatocytes (Martınez-Flores *et al.* 2005). Hamalainen *et al.* 2007 stated that quercetin and kaempferol which able to inhibit the activation of NF–kB and STAT–1 in activated macrophage are likely to down–regulate production of an array of inflammatory mediators in addition to iNOS. In Chang Liver cells which the effect of quercetin and kaempferol were found inhibited the activation of NFkB that induced by a cytokine mixture (Garcıa Mediavilla *et al.* 2007; Hamalainen *et al.* 2007). Hejinen *et al.* (2001) reported that the optimal configuration for free radical scavenging scavenging activity of quercetin was derived from the presence of two antioxidant pharmacophores within the molecule which are catechol group in the B ring and the OH group at position 3 of the AC ring whereas kaempferol was due to AC ring with three OH groups. The presence of 4–oxo–3–hydroxyl in kaempferol attributed in NO scavenging activity. On the other hand, there are reports of a variety of bioactivities of caffeoylquinic acids including antioxidant and anti – inflammatory (Zhao *et al.* 2006; Hung *et al.* 2006). Phenolic compounds possessing catechol structures, such as 5–O–caffeoylquinic acid, scavenge the nitric oxide ion and peroxynitrite ion by electron donation, generating the corresponding quinone (Choi *et al.* 2002; Rodrigues *et al.* 2014). Eventually, although the present result only revealed the ability of extracts from *P.rubra* flower to scavenge NO as the oxidative stress

in *in vitro* model but at least the potential of bioactive compound also been reported to be effective in inflammatory mediator in *in vivo* model.

5.4.6 Superoxide Radical Scavenging Activity of *P. rubra* Flower Extracts

Superoxide anion itself is not considered as strong oxidant, however, it is responsible for the formations of other active reactive species such as hydrogen peroxide, peroxynitrite, and hydroxyl radicals (Stief, 2003). In this assay, superoxide radicals are generated in non-enzymatic system, PMS/NADH system, and acts as reducing agents of NBT. The superoxide radical scavenging assay evaluated the capability of extracts from red flowers of *P. rubra* to scavenge superoxide radicals as well as diminish the reduction of NBT. The reduction of NBT in presence of antioxidants was measured. The decrease of absorbance at 570 nm upon the addition of extracts as well as standard quercetin and ascorbic acid, is the indication of consumption of superoxide anion in the reaction mixture. Superoxide anion is a reduced form of molecular oxygen created by receiving one electron. Superoxide radicals have been observed to kill cells, inactivate enzymes, and degrade DNA, cell membranes and polysaccharides (Halliwell, 1996; Stief, 2003).

The superoxide scavenging activity of extracts from *P. rubra* red flowers was increased markedly with the increase in concentrations. The present study shows that all the extracts from *P. rubra* flowers are potentially act as superoxide scavenger except hexane extract. Moreover, good inhibitory effects of the PR-ME of *P.rubra* on superoxide anion formation noted herein possibly render it as a promising antioxidants. The standard ascorbic acid showed the most effective of scavenging activity against superoxide radical. The highest scavenging activity against superoxide radical in PR−ME was due to the presence of phytochemical compounds which detected by LCMS/MS analysis. Several of these compounds has been reported from previous research as superoxide scavenger which including phenolic acids (3−O−caffeoylquinic acid, 5−O−caffeoylquinic acid, quinic

acid and also mixture of flavonoids compound such as quercetin, kaempferol and rutin. The hydroxyl group at C−3' and C−4'of the B ring in rutin, kaempferol and quercetin should be contributed for superoxide radical scavenging activity due to hydrogen donation activity (Wang *et al.* 2010). Nevertheless, the efficiency of superoxide scavenging could be decreased by blocking of hydroxyl groups by sugar or alkoxyl substituents through glycosylation of quercetin and or kaempferol (Calderon-Montano *et al.* 2011). Surprisingly, the present study indicated that PR−ME was more efficient in scavenging of superoxide anion activity compared to standard quercetin which revealed the synergistic effect of superoxide scavenging activity from phenolic (3−O−caffeoquinic acid, 5−O−caffeoquinic acid, quinic acid) and flavonoid (quercetin, kaempferol, rutin) compounds.

5.5 Xanthine Oxidase (XO) Inhibitory Activity of *P.rubra* Flower Extracts

The studies of XO inhibitory activity in extracts from *P. rubra* red flowers was evaluated in order to evaluated its effectiveness in reducing the production of uric acid. Allopurinol which was used as positive standard had been widely used as standard positive reference due to its excellent action in inhibiting activity of XO enzyme through in *in vitro* assay and also in *in vivo* experimental animal model (Umamaheswari *et al.* 2007; Wang *et al.* 2008; Murugaiyah & Chan, 2009; Wang *et al.* 2010; Chen *et al.* 2011; Liu *et al.* 2014). As the XO enzyme was inhibited, it reduced the XO activity and this would then eventually inhibit the catalyzation of hypoxanthine to xanthine, then xanthine to uric acid. Thus, the inhibition of XO enzyme will reduce the production of uric acid.

5.5.1 XO Inhibitory in *In Vitro* Model

The highest of XO inhibition in *in vitro* assay shown in PR-ME and this could be due to the presence of polyphenols such as $3-O-$caffeolquinic acid and chlorogenic acid while flavonoids such as quercetin, kaempferol and rutin as these phytochemicals has been detected by LCMS/MS. As reported by several researches, flavonoids such as rutin, kaempferol and quercetin has a high potential in order to inhibite the activity of XO enzyme (Cos *et al.* 1998, Nagao *et al.* 1999; Selloum *et al.* 2001; Ahmad *et al.* 2008; Matsuoka *et al.* 2012). Therefore, it can be concluded that the presence of these compounds in PR-ME could contributed to the inhibition of XO activity. On the other hand, Masuoka *et al.* (2012) described that XO inhibition activity of kaempferol $3-O$ glucosides was lower compared to flavonoid aglycone due to these flavonoids competitively bound a xanthine binding site in xanthine oxidase instead of the flavonoid glycosides. In addition, Cos *et al.* (1998) stated that the chemical structure of flavonoids contain of hydroxyl groups at C5 and C7 and the double bond between C2 and C3 were essential for a high inhibitory activity on xanthine oxidase. Later on, Nagao *et al.* (1999) stated hydroxyl group at C5 and C7 of flavonoid was essential for inhibition of xanthine oxidase activity but not a hydroxyl group at C3 of the flavone structure such as quercetin. Otherwise, quercetin which have hydroxyl group at C3' and C4' of the B ring showed slightly higher XO inhibition activity than kaempferol $(3'-OH)$. Catechol structure in the B ring which gives the antioxidant potential to flavonoids was not related to the xanthine oxidase inhibition (Nagao *et al.* 1999). Recently, Wang *et al.* (2015) stated that the main inhibition mechanism of kaempferol on XO enzyme could be due to the insertion of kaempferol into the active site of XO occupying the catalytic center of the enzyme then avoid the entrance of the substrate and inducing conformational changes of XO. Furthermore, kaempferol also capable to inhibited XO activity and additional superoxide scavenging activity (Cos *et al.* 1998). In addition, the results showed that the

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XO inhibition activity could be linked to the content and the nature of flavonoids and phenolic compounds contained in the extracts, and eventually to the presence of other types of molecules (Salem *et al.* 2011). In fact, the present result was an agreement with other researchers that possible synergy between polyphenols and other components present in methanol extracts might contributed to their overall antioxidant activity (Shahidi *et al.* 1994; Ordonez *et al.* 2006).

Generally, the present study indicated that PR−ME of red flowers from *P. rubra* produced the highest activity on both XO inhibition activity and also superoxide radical scavenging assay. In fact, the inhibition activity of XO enzyme was higher compared to superoxide scavenging activity. Thus, the PR−ME from *P.rubra* could be categorized as XO inhibitors with additional superoxide scavenging activity as reported by Cos *et al.* (1998). On the other hand, Matsuoka *et al.* (2012) had summarized the three possible mechanism pathway in order to be an antioxidant to superoxide radicals and inhibited XO activity. These include (A) with the inhibition of XO enzyme, antioxidants inhibit uric acid formation and this inhibition suppresses both superoxide generation and also hydrogen peroxide formation. Apart from that, with the reduction reaction of XO (B), antioxidants act as a reducing reagent of XO. The reduced XO enzyme only catalyses the hydrogen peroxide formation. Antioxidants also function by means of the superoxide scavenging activity (C) and the activity could be monitored by using the PMS−NADH system.

PR−ME showed strongest XO inhibition activity in *in vitro* model and this is strongly correlate with its ethnomedicinal used the trearment of inflammatory disorder and rheumatic disease (Dubois & Rezzonico, 2007; Kumar *et al.* 2009), and it was furthered investigated for its capability to reducing uric acid in serum of induced

hyperuricemic rats as well as evaluated the XO inhibition activity in serum and liver of induced hyperuricemic rats.

5.5.2 XO Inhibitory Activity in *In Vivo* Model

Acute toxicity study indicated the non-toxic nature of the *P.rubra* extracts. There was no lethality or any toxic reactions found at both doses selected, 2000 mg/kg and 4000 mg/kg body weight of PR–ME extract until the end of the study period.

In animal study, the anti–hyperuricemic activity and XO inhibitory activity of PR–ME of red flower from *P. rubra* were evaluated in *in vivo* model with the doses of 200 mg/kg and 400 mg/kg body weight. The allopurinol is used as standard and the results showed that body weight of treatment group fed with dose of 10 mg/kg body weight showed no significant different to all groups of study. Furthermore, XO inhibition activity were determined in both within serum and liver of PR–ME and these were also carried out in *in vivo* with the doses of 200mg/kg and 400mg/kg body weight. The body weights of eight groups increased gradually through 7 days treatment, and no significant differences (p < 0.05) in body weights among eight groups were observed. In addition, the rats in each group had lustrous body hair, normal water consumption, diet consumption, and urine volume and were in good mental state during the experimental period, indicating that PR–ME of *P. rubra* flowers did not exhibited any side effects on the rats. The allopurinol was used as standard and the result showed that body weight of group 8, which group of rats were induced intraperitoneally with PO then fed with allopurinol showed no significant differences with untreated normal group as well as treated group of both doses, 200 mg/kg and 400 mg/kg.

Result demonstrated that PR–ME was significantly exert serum urate levels in hyperuricemic induced rat but not in normal rats after 7 days of treatment. Both doses of PR–ME (400 mg/kg and 200 mg/kg) shown to elicit a dose-dependent decrease

in serum uric acid levels only in hyperuricaemic induced rats. This finding was similar to the previous research by that most of medicinal plant extract exhibited less inhibitory effects on serum uric acid levels in normal mice compared with those animals preinduced with PO (Kong *et al.*, 2004; Wang *et al.*, 2004; Zhu *et al.*, 2004; Zhao *et al.*, 2006). Thus, the detected phytochemical compounds in methanol extract by LCMS/MS analysis did not exhibited significant reduction on uric acid levels in normal rats within 7 days of administration. In addition, result from normal rats indicated that PR–ME from *P. rubra* flower was safe as XO inhibitor compared than allopurinol in treatment of hyperuricemia. Moreover, the clinical use of both dose PR–ME from *P. rubra* flower in the treatment of gout and hyperuricemia might be safer than that of allopurinol. Despite Nuki (2006) has found that the elevated levels of uric acid in the circulation could give rise to gout and possibly other pathological conditions, Stinefelt (2005) has gave the different theory whereby the antioxidant action of uric acid, particularly its ability to inhibit DNA damage, is also well proven. Thus, excessive lowering of the uric acid level in the circulation beyond that of the normal range might even be counterproductive (Wang *et al.* 2004).

The serum urate level of hyperuricemic induced rats with doses of 200 and 400 mg/kg of PR–ME of *P. rubra* flower were decreased significantly in a dose dependent manner. The uric acid lowering effect of group 6, which was hyperuricemic induced rats treated with high dose of 400 mg/kg PR–ME and group 7 which was hyperuricemic induced rats treated with low dose of 200 mg/kg PR–ME indicated that the PR–ME of *P. rubra* flower has the potential to attenuate hyperuricemia. As compared to standard, allopurinol, known as a protective role in XO inhibitor showed most effective hypouricemic action in *in vivo* study with 78.2 % of uric acid lowering effect even in a dose only 10 mg/kg. The decreasing of uric acid levels in serum could be due to the presence of compounds which including quercetin, kaempferol and rutin which contaim in PR – ME of *P.rubra* flowers as detected in LCMS analysis. In fact, kaempferol,

quercetin and rutin were capable of reducing the accumulation of purine metabolites in blood following PO–induction rats as reported by several literatures (Zhu *et al.* 2004; Rashidi *et al.* 2008; Chen *et al.* 2011; Mathew *et al.* 2015).

The XO inhibition activity of PR–ME from *P.rubra* flower was carried out in *in vivo* model with the doses of 200 mg/kg and 400 mg/kg body weight. It should be noted that the activity of XO enzyme in serum and liver at normal mechanism was not influenced with this form of dose (400 mg/kg). Thus, this high dose (400 mg/kg) did not have allopurinol–like hypouricemic effects influencing uric acid levels in the normal purine metabolic system. In the contrast low dose (200 mg/kg) has a different effect that increased the activity of XO enzyme in serum but not in liver. For this reason, 200 mg/kg of this extract was not suggested dose to be administered when hyperuricemia was not triggered. The varies result of XO activity in normal rats could be due to different effect of reaction from different phytochemical compounds presence in PR – ME which comprising of phenols such as 3 – O – caffeoylquinic acid and chlorogenic acid while flavonoids such as quercetin, kaempferol and rutin and flavonoid as detected from LCMS/MS. Despite that previous literature reported that the good natural products previously indicated alteration in uric acid levels and XO activity occurs only in the hyperuriceamic mice, but not in the normal mice, the capability of PR–ME extract cannot be excluded.

The inhibition effects of XO activity was more dominants in groups of hyperuricemia–induced rats compared to their effects on normal rats. Moreover, the XO enzyme activity in serum was correlated with XO enzyme activity in liver within hyperuricemic –induced rats. According to Yoshisue *et al.* (2000), XO is well known of inducible enzyme, thus, PR–ME was found exert the inhibitory action on induced XO in both serum and liver of hyperuricemic-induced rats in a dose – dependent manner.

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Accordingly, it could be possible that the presence of phenols and flavonoids which including 3–O–caffeolquinic acid, 5–O–caffeoylquinic acid, chlorogenic acid, kaempferol, quercetin and rutin may partly contribute to the beneficial effects of PR–ME extract on the inhibition of XO enzyme activities. In addition, Zhu *et al.* (2004) described that quercetin and rutin were capable to inhibit XO enzyme in *in vitro* and *in vivo* model. Thus, the hypouricemic effect produced by PR–ME extract of *P. rubra* might be contributing from the phytochemical compound such as quercetin, rutin. Moreover, kaempferol seem to be partly mediated by the inhibition XO activities and was better inhibitor of xanthine/xanthine oxidase than the two glycosides (Wang *et al.* 2015; Haidari *et al.* 2011). Nevertheless, the standard XO inhibitor, allopurinol at dose of 10 mg/kg produced stronger inhibition effect of XO enzyme in both of serum and liver, as reported as several researches (Zhu *et al.* 2004; Wang *et al.*2008; Chen *et al.*,2011; Huang *et al.* 2011; Haidari *et al.* 2011; Lima *et al.* 2015).

The present results indicated that the PR–ME of red flowers from *P. rubra* possess good antioxidant activity via different mechanisms including hydrogen atom donation, ability in reducing ferric ions, metal chelating effect, hydrogen peroxide scavenging ability, nitric oxide scavenging ability and superoxide anion scavenging ability. These antioxidant properties of the PR–ME were attributed to the presence of phenolics and flavonoids compound as detected in LCMS/MS. Moreover, the PR–ME showed potential of XO inhibitory activity in *in vitro* assay as well as in *in vivo* in both serum and liver of PO–induced hyperuricemic rats. Furthermore, reducing effects of serum urate levels from PO–induced hyperuricemic was mediated to XO inhibitory activity of PR–ME. Besides, there are relation between oxidative stress and gout disease, as XO enzyme contributed to production of uric acid by catalyzing hypoxanthine to xanthine, with concomitant production of superoxide and hydrogen peroxide radicals

(Dalbeth & Haskard, 2005; Umamaheswari *et al.* 2007; Kong *et al.* 2000). Thus, the results of this research has shown that the bioactive compounds detected in the PR−ME from *P. rubra* flowers capable to inhibited the activity of XO enzyme as play direct role to hyperuricemia and disease such as gout, endothelial dysfunction, atherosclerosis as well as indirect role as a source of ROS.

CHAPTER 6

CONCLUSION

The phytochemical bioactive compounds of *Plumeria rubra* Linn red flowers was detected using Thin Layer Chromatography (TLC) and displayed the presence of flavonoids, phenols, terpenoids and essential oils while detected with chemical reagents. The analysis of the methanol (PR–ME) and water (PR–WE) extract of *P.rubra* with Liquid Chromatography Mass Spectrometer tandem mass spectrometer (LCMS/MS) indicated that the presence contains 3–caffeoylquinic acid, 5–caffeoylquinic acid, 1, 3–dicaffeoquinic acid, chlorogenic acid, citric acid, 3,3–di–O methylellagic acid, kaempferol–3–O–glucoside, kaempferol–3–rutinoside, kaempferol, quercetin 3–O–α–L–arabinopyranoside, quercetin, quinic acid and rutin. Both of the contents of TPC and TFC showed the PR–ME extract from red flowers of *P. rubra* contained the highest phenols and flavonoids content. The PR–ME extract of red flower from *P. rubra* has the highest XO inhibitory activity in *in vitro*, and therefore it was selected for further in *in vivo* model. The oral acute toxicity test revealed that the PR–ME extract of *P. rubra* red flower was nontoxic nature at the dose of 4000 mg/kg, and no lethality or any toxic effects was observed during 14 days of monitoring. Furthermore, PR–ME extract of *P. rubra* red flower could reduced serum uric acid level in dose dependent manner in hyperuricemic rats induced by PO but not in the normal rats within 7 days experiment. Along with the good XO inhibitory activity in *in vitro* assay, the PR–ME extract of *P. rubra* red flower also exhibited inhibition activity against XO enzyme in serum and liver in a dose–dependent manner within 7 days experiment. Moreover, no significant difference of body weight between treated rats and normal rats within 7 days of extract administration. The

antihyperuricemic activity of the PR−ME extract from *P. rubra* red flowers seems to be mediated by XO inhibition. In the six differents of antioxidant activity assay, PR−ME extract of *P. rubra* red flowers demonstrated highest in DPPH scavenging activity, ferric reducing power, chelating activity, hydrogen peroxide radical scavenging activity, nitric oxide radical scavenging activity and also superoxide radicals scavenging activity. Apparently, the inhibition activity of XO enzyme in *in vitro* and in i*n vivo* model, antihyperuricemic in serum and antioxidant properties of red flowers from *P. rubra* were due to presence of phytochemical compounds that has been detected and identified by in TLC and LCMS/MS methods. Thus, this study indicate that red flower of *P. rubra* is useful for new therapeutic option of management of gout or other XO− induced diseases.

REFERENCES

Abadeh, S., Killacky, J., Benboubetra, M., & Harrison, R. (1992). Purification and partial characterization of xanthine oxidase from human milk. *Biochimica et Biophysica Acta (BBA)– General Subjects*, *1117*(1): 25–32.

Afonso, V., Champy, R., Mitrovic, D., Collin, P., & Lomri, A. (2007). Reactive Oxygen Species and Superoxide Dismutases: Role in Joint Diseases. *Joint Bone Spine*, *74*(4): 324–329.

Aggarwal, B. B., Bhardwaj, A., Aggarwal, R. S., Seeram, N. P., Shishodia, S., & Takada, Y. (2004). Role of Resveratrol in Prevention and Therapy of Cancer: Preclinical and Clinical Studies. *Anticancer Research*, *24*(5A): 2783–2840.

Aggarwal, B. B., & Shishodia, S. (2006). *Resveratrol in health and disease*. Boca Raton: CRC Taylor & Francis.

Agudelo, C. A. (1989). Gout and Hyperuricemia. *Current Opinion in Rheumatology*, *1*(3) :286–293.

Ahern M., Reid C., Gordon T., McCredie M., Brooks P., Jones M. (1987) Does colchicine work? The results of the first controlled study in acute gout. *Australian and New Zealand Journal of Medicine 17*(3): 301–304.

Ahmad, N. S., Farman, M., Najmi, M. H., Mian, K. B., & Hasan, A. (2008). Pharmacological Basis for Use of *Pistacia integerrima* Leaves in Hyperuricemia and Gout. *Journal of Ethnopharmacology*, *117*(3): 478–482.

Akhtar, N., Saleem, M., Riaz, N., Ali, M. S., Yaqoob, A., Nasim, F., & Jabbar, A. (2013). Isolation and characterization of the chemical constituents from *Plumeria rubra*. *Phytochemistry Letters*, *6*(2): 291–298.

Andjelković, M., Van Camp, J., De Meulenaer, B., Depaemelaere, G., Socaciu, C., Verloo, M., & Verhe, R. (2006). Iron-chelation properties of phenolic acids bearing catechol and galloyl groups. *Food Chemistry*, *98*(1): 23–31.

Apak, R., Güçlü, K., Demirata, B., Özyürek, M., Çelik, S. E., Bektaşoğlu, B., & Özyurt, D. (2007). Comparative evaluation of various total antioxidant capacity assays applied to phenolic compounds with the CUPRAC assay. *Molecules*, *12*(7):1496–1547.

Apati, P., Szentmihályi, K., Kristo, S., T., Papp, I., Vinkler, P., Szoke, E., & Kery, A. (2003). Herbal remedies of Solidago—correlation of phytochemical characteristics and antioxidative properties. *Journal of Pharmaceutical and Biomedical Analysis*, *32*(4): 1045–1053.

Avram, Z., & Krishnan, E. (2008). Hyperuricaemia--where nephrology meets rheumatology. *Rheumatology*, *47*(7): 960-964.

Aziz, A., Mutheerah, S., Low, C. N., Chai, L. C., Razak, A., Nazfah, S. S., ... & Khatib, A. (2011). Screening of Selected Malaysian Plants Against Several Food Borne Pathogen Bacteria. *International Food Research Journal*, *18*(3): 1195–1201.

Azmi, S. M. N., Jamal, P., & Amid, A. (2012). Xanthine oxidase inhibitory activity from potential Malaysian medicinal plant as remedies for gout. *International Food Research Journal*, *19*(1): 159–165.

Balunas, M. J., & Kinghorn, A. D. (2005). Drug Discovery from Medicinal Plants. *Life sciences*, *78*(5): 431–441.

Baroletti, S., Bencivenga, G., & Gabardi, S. (2004). Treating gout in kidney transplant recipients. *Progress in Transplantation*, *14*(2): 143–147.

Barreira, J. C. M., Ferreira, I. C. F. R., Oliveira, M. B. P. P., & Pereira, J. A. (2008). Antioxidant activities of the extracts from chestnut flower, leaf, skin and fruit. *Food Chemistry*, *107*: 1106–1113.

Becker, M. A., Schumacher, H. R., Espinoza, L. R., Wells, A. F., Macdonald, P., Lloyd, E., & Lademacher, C. (2010). The urate-lowering efficacy and safety of febuxostat in the treatment of the hyperuricemia of gout: the CONFIRMS trial. *Arthritis Research & Therapy*, *12*(2).

Becker, M. A., Schumacher, H. R., Wortmann, R. L., Macdonald, P. A., Eustace, D., Palo, W. A., . . . Joseph-Ridge, N. (2005). Febuxostat Compared with Allopurinol in Patients with Hyperuricemia and Gout. *New England Journal of Medicine*, *353*(23): 2450–2461.

Benzie, I. F., & Strain, J. J. (1996). The Ferric Reducing Ability of Plasma (FRAP) as A Measure of "Antioxidant Power": the FRAP assay. *Analytical biochemistry, 239*(1): 70–76.

Berry, C. E., & Hare, J. M. (2004, March 16). Xanthine oxidoreductase and cardiovascular disease: molecular mechanisms and pathophysiological implications. *The Journal of Physiology*, *555*(Pt 3): 589–606.

Bezerra, R. J. S., Calheiros, A. S., Ferreira, N. C. S., Frutuoso, V. S., Alves, L. A., (2013) Natural products as a source for new anti-inflammatory and analgesic compounds through the inhibition of purinergic P2X receptors. *Pharmaceuticals (Basel) 6*(5): 650–658.

Biglari F., AlKarkhi A. F., Easa A. M. (2008) Antioxidant Activity and Phenolic Content of Various Date Palm (*Phoenix dactylifera*) Fruits from Iran. *Food Chemistry 107*(4): 1636–1641.

Bimakr, M., Rahman, R. A., Taip, F. S., Ganjloo, A., Salleh, L. M., Selamat, J., & Zaidul, I. S. M. (2011). Comparison of different extraction methods for the extraction of major bioactive flavonoid compounds from spearmint (*Mentha spicata L.)* leaves. *Food and Bioproducts Processing, 89*(1): 67–72.

Bobbarala, V., Katikala, P. K., Naidu, K. C., & Penumajji, S. (2009). Antifungal activity of selected plant extracts against phytopathogenic fungi *Aspergillus niger* F2723. *Indian Journal of Science and Technology*, *2*(4): 87–90.

Borges, F., Fernandes, E., & Roleira, F. (2002). Progress Towards the Discovery of Xanthine Oxidase Inhibitors. *Current Medicinal Chemistry*, *9*(2): 195–217.

Boumerfeg, S., Baghiani, A., Messaoudi, D., Khennouf, S., & Arrar, L. (2009). Antioxidant Properties and Xanthine Oxidase Inhibitory Effects of *Tamus communis* L. Root Extracts. *Phytotherapy Research*, *23*(2): 283–288.

Brighente, I. M. C., Dias, M., Verdi, L. G., & Pizzolatti, M. G. (2007). Antioxidant Activity and Total Phenolic Content of Some Brazilian Species. *Pharmaceutical Biology*, *45*(2): 156–161.

Burkill, I. H. (1966). *A dictionary of the economic products of the Malay Peninsula*. Kuala Lumpur, Malaysia: Ministry of Agriculture and Co-operatives.

Byamukama, R., Namukobe, J., Jordheim, M., Andersen, Ø. M., & Kiremire, B. T. (2011). Anthocyanins from ornamental flowers of red frangipani, *Plumeria rubra*. *Scientia horticulturae*, *129*(4): 840–843.

Caldeira, J., Belle, V., Asso, M., Guigliarelli, B., Moura, I., Moura, J. J., & Bertrand, P. (2000). Analysis of the Electron Paramagnetic Resonance Properties of the [2Fe-2S]1 Centers in Molybdenum Enzymes of the Xanthine Oxidase Family: Assignment of Signals I and II. *Biochemistry*, *39*(10): 2700–2707.

Calderon-Montano, J. M., Burgos-Moron, E., Perez-Guerrero, C., & Lopez-Lazaro, M. (2011). A Review on the Dietary Flavonoid Kaempferol. *Mini-Reviews in Medicinal Chemistry*, *11*(4): 298–344.

Cammalleri, L., & Malaguarnera, M. (2007). Rasburicase represents a new tool for hyperuricemia in tumor lysis syndrome and in gout. *International Journal of Medical Sciences*, *4*(2): 83–93.

Cassel, S. L., Eisenbarth, S. C., Iyer, S. S., Sadler, J. J., Colegio, O. R., Tephly, L. A., … Sutterwala, F. S. (2008). The Nalp3 inflammasome is essential for the development of silicosis. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(26): 9035–9040.

Caulfield, M. J., Munroe, P. B., O'neill, D., Witkowska, K., Charchar, F. J., Doblado, M., . . . Cheeseman, C. (2008). SLC2A9 Is a High-Capacity Urate Transporter in Humans. *PLoS Medicine*, *5(*10).

Chanda, S. V., & Nagani, K. V. (2010). Antioxidant Capacity of *Manilkara zapota L.* Leaves Extracts Evaluated by Four *in vitro* Methods. *Nature and Science*, *8*(10): 260–266.

Chen, J., Ho, F., Chao, P. L., Chen, C., Jeng, K. G., Hsu, H., . . . Lin, W. (2005). Inhibition of iNOS gene expression by quercetin is mediated by the inhibition of IκB kinase, nuclear factor-kappa B and STAT1, and depends on heme oxygenase-1 induction in mouse BV-2 microglia. *European Journal of Pharmacology*, *521*(1-3): 9–20.

Chen, L., Yin, H., Lan, Z., Ma, S., Zhang, C., Yang, Z., & Lin, B. (2011). Anti-hyperuricemic and Nephroprotective Effects of *Smilax china L. Journal of Ethnopharmacology*, *135*(2): 399–405.

Chiang, H. C., Lo, Y. J., & Lu, F. J. (1994). Xanthine Oxidase Inhibitors from the Leaves of *Alsophila spinulosa* (Hook) Tryon. *Journal of Enzyme Inhibition*, *8*(1): 61–71.

Chin, H. F. (1986). *Malaysian flowers in colour*. Kuala Lumpur: Tropical Press.

Choi, H. K. (2005). Pathogenesis of Gout. *Annals of Internal Medicine*, *143*(7): 499.

Choi, H. K., Atkinson, K., Karlson, E. W., Willett, W., & Curhan, G. (2004). Alcohol intake and risk of incident gout in men: a prospective study. *The Lancet*, *363*(9417): 1277–1281.

Choi, H. K., Atkinson, K., Karlson, E. W., Willett, W., & Curhan, G. (2004). Purine-Rich Foods, Dairy and Protein Intake, and the Risk of Gout in Men. *New England Journal of Medicine*, *350*(11): 1093–1103.

Choi, H. K., & Curhan, G. (2008). Soft drinks, fructose consumption, and the risk of gout in men: prospective cohort study. *Bmj*, *336*(7639): 309–312.

Choi, H. K., Ford, E. S., Li, C., & Curhan, G. (2007). Prevalence of the metabolic syndrome in patients with gout: *The Third National Health and Nutrition Examination Survey. Arthritis & Rheumatism*, *57*(1): 109-115.

Choi, H. K., Vera, M. A., & Krishnan, E. (2008). Gout and the risk of type 2 diabetes among men with a high cardiovascular risk profile. *Rheumatology*, *47*(10): 1567–1570.

Choi, J. S., Chung, H. Y., Kang, S. S., Jung, M. J., Kim, J. W., No, J. K., & Jung, H. A. (2002). The structure–activity relationship of flavonoids as scavengers of peroxynitrite. *Phytotherapy Research, 16*(3): 232–235.

Chvátalová, K., Slaninová, I., Březinová, L., & Slanina, J. (2008). Influence of dietary phenolic acids on redox status of iron: Ferrous iron autoxidation and ferric iron reduction. *Food Chemistry, 106*(2): 650–660.

Cimanga, R. K., Mukenyi, P. N. K., Kambu, O. K., Tona, G. L., Apers, S., Totte, J., & Vlietinck, A. J. (2010). The spasmolytic activity of extracts and some isolated compounds from the leaves of *Morinda morindoides* (Baker) *Milne-Redh*.(Rubiaceae). *Journal of Ethnopharmacology, 127*(2): 215–220.

Cleland, L. G., Hill, C. L., & James, M. J. (1995). Diet and arthritis. *Baillière's clinical rheumatology, 9*(4): 771–785.

Corner, E. J. (1997). *Wayside trees of Malaya*. Kuala Lumpur: Malayan Nature Society.

Corrado, A., D'Onofrio, F., Santoro, N., Melillo, N., & Cantatore, F.P. (2006). Pathogenesis, clinical findings and management of acute and chronic gout. *Minerva medica, 97*(6): 495–509.

Cos, P., Ying, L., Calomme, M., Hu, J. P., Cimanga, K., Van Poel, B., & Berghe, D. V. (1998). Structure-activity relationship and classification of flavonoids as inhibitors of xanthine oxidase and superoxide scavengers. *Journal of Natural Products, 61*(1):71–76.

Dai, J., & Mumper, R. J. (2010). Plant Phenolics: Extraction, Analysis and Their Antioxidant and Anticancer Properties. *Molecules, 15*(10): 7313–7352.

Dalbeth, N. (2005). Mechanisms of inflammation in gout. *Rheumatology, 44*(9): 1090–1096.

Dalbeth, N., Clark, B., Gregory, K., Gamble, G., Sheehan, T., Doyle, A., & Mcqueen, F. M. (2008). Mechanisms of bone erosion in gout: a quantitative analysis using plain radiography and computed tomography. *Annals of the Rheumatic Diseases, 68*(8): 1290–1295.

Dalbeth, N., & Merriman, T. (2008). Crystal ball gazing: new therapeutic targets for hyperuricaemia and gout. *Rheumatology*, *48*(3): 222–226.

De Souza, M. R., de Paula, C. A., de Resende, M. L. P., Grabe-Guimarães, A., de Souza Filho, J. D., & Saúde-Guimarães, D. A. (2012). Pharmacological basis for use of *Lychnophora trichocarpha* in gouty arthritis: Anti-hyperuricemic and anti-inflammatory effects of its extract, fraction and constituents. *Journal of Ethnopharmacology*, *142*(3): 845–850.

Doherty, M. (2009). New insights into the epidemiology of gout. *Rheumatology, 48*(Suppl 2): Ii2-Ii8.

Dubchak, N., & Falasca, G. F. (2010, November 24). New and improved strategies for the treatment of gout. *International Journal of Nephrology and Renovascular Disease*, *3*:145–166.

Dubois, M.G., Rezzonico, B., (2007). Pentacyclic triterpenes from *Plumeria inodora*. *Chemistry of Natural Compounds*, *43*(3): 351–352.

Elahi, M. M., Kong, Y. X., & Matata, B. M. (2009). Oxidative stress as a mediator of cardiovascular disease. *Oxidative Medicine and Cellular Longevity*, *2*(5): 259–269.

Elion, G. (1989). The purine path to chemotherapy. *Science, 244*(4900): 41–47.

Enomoto, A., Kimura, H., Chairoungdua, A., Shigeta, Y., Jutabha, P., Cha, S. H., & Matsuo, H. (2002). Molecular identification of a renal urate–anion exchanger that regulates blood urate levels. *Nature*, *417*(6887): 447–452.

Enroth, C., Eger, B. T., Okamoto, K., Nishino, T., Nishino, T., & Pai, E. F. (2000). Crystal structures of bovine milk xanthine dehydrogenase and xanthine oxidase: Structure-based mechanism of conversion. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(20): 10723–10728.

Facchini, F. (1991). Relationship Between Resistance to Insulin-Mediated Glucose Uptake, Urinary Uric Acid Clearance, and Plasma Uric Acid Concentration. *Jama*, *266*(21): 3008.

Farkas, L., Gábor, M., & Kállay, F. (1986). *Flavonoids and bioflavonoids, 1985: Proceedings of the 7th Hungarian Bioflavinoid Symposium, Szeged, Hungary, May 16–18, 1985*. Amsterdam: Elsevier.

Fathallah-Shaykh, S. A., & Cramer, M. T. (2014). Uric acid and the kidney. *Pediatric Nephrology*, *29*(6): 999–1008.

Fernandez, M. T., Mira, M. L., Florencio, M. H., & Jennings, K. R. (2002). Iron and copper chelation by flavonoids: an electrospray mass spectrometry study. *Journal of Inorganic Biochemistry*, *92*(2): 105–111.

Ferreres, F., Pereira, D. M., Valentão, P., Andrade, P. B., Seabra, R. M., & Sottomayor, M. (2008). New Phenolic Compounds and Antioxidant Potential of *Catharanthus roseus*. *Journal of Agricultural and Food Chemistry*, *56*(21): 9967–9974.

Fields, M., Lewis, C. G., & Lure, M.D. (1996). Allopurinol, an inhibitor of xanthine oxidase, reduces uric acid levels and modifies the signs associated with copper deficiency in rats fed fructose. *Free Radical Biology and Medicine 20* (4): 595–600.

Firuzi, O., Lacanna, A., Petrucci, R., Marrosu, G., & Saso, L. (2005). Evaluation of the antioxidant activity of flavonoids by "ferric reducing antioxidant power" assay and cyclic voltammetry. *Biochimica et Biophysica Acta (BBA)-General Subjects*, *1721*(1): 174–184.

Frishman, W. H., Beravol, P., & Carosella, C. (2009). Alternative and Complementary Medicine for Preventing and Treating Cardiovascular Disease. *Disease-a-Month*, *55*(3): 121–192.

Gao, X., Qi, L., Qiao, N., Choi, H. K., Curhan, G., Tucker, K. L., & Ascherio, A. (2007). Intake of added sugar and sugar-sweetened drink and serum uric acid concentration in US men and women. *Hypertension, 50*(2): 306–312.

García-Mediavilla, V., Crespo, I., Collado, P. S., Esteller, A., Sánchez-Campos, S., Tuñón, M. J., & González-Gallego, J. (2007). The anti-inflammatory flavones quercetin and kaempferol cause inhibition of inducible nitric oxide synthase, cyclooxygenase-2 and reactive C-protein, and down-regulation of the nuclear factor kappaB pathway in Chang Liver cells. *European Journal of Pharmacology*, *557*(2): 221–229.

Gariboldi, E., Mascetti, D., Galli, G., Caballion, P., & Bosisio, E. (1998). LC-UV-electrospray-MS-MS mass spectrometry analysis of plant constituents inhibiting xanthine oxidase. *Pharmaceutical Research*, *15*(6): 936–943.

Ghafar, M. F., Prasad, K. N., Weng, K. K., & Ismail, A. (2010). Flavonoid, hesperidine, total phenolic contents and antioxidant activities from Citrus species. *African Journal of Biotechnology, 9*(3): 326–330.

Godber, B. L., Schwarz, G., Mendel, R. R., Eisenthal, R., & Harrison, R. (2005). Molecular characterization of human xanthine oxidoreductase: the enzyme is grossly deficient in molybdenum and substantially deficient in iron-sulphur centres. *Biochemical Journal*, *388*(2): 501–508.

Goli, A. H., Barzegar, M., & Sahari, M. A. (2005). Antioxidant activity and total phenolic compounds of pistachio *(Pistachia vera*) hull extracts. *Food Chemistry*, *92(3*): 521–525.

González, A. G., Bazzocchi, I. L., Moujir, L., Ravelo, A. G., Correa, M. D. and Gupta, M. P. (1995). Xanthine Oxidase Inhibitory Activity of Some Panamanian Plants from celastraceae and lamiaceae. *Journal of Ethnopharmacology 46* (1): 25–29.

Goodman, L. S., Gilman, A., Brunton, L. L., Lazo, J. S., & Parker, K. L. (2006). *Goodman & Gilman's the Pharmacological Basis of Therapeutics*. New York: McGraw-Hill.

Gordon M.H. (1990). *The mechanism of antioxidant action in vitro. In Food antioxidants*. Springer Netherlands.

Gupta, M., Mazumder, U., Gomathi, P., & Selvan, V. T. (2006). Antiinflammatory evaluation of leaves of *Plumeria acuminata*. *BMC Complementary and Alternative Medicine*, *6*(1): 1.

Haddad, J. J. (2002). Antioxidant and prooxidant mechanisms in the regulation of redox(y)-sensitive transcription factors. *Cellular Signalling*, *14*(11): 879–897.

Haenen, G. R., & Bast, A. (1999). [50] Nitric oxide radical scavenging of flavonoids. *Methods in Enzymology*, *301*: 490–503.

Haidari, F., Keshavarz, S. A., Rashidi, M. R., & Shahi, M. M. (2009). Orange Juice and Hesperetin Supplementation to Hyperuricemic Rats Alter Oxidative Stress Markers and Xanthine Oxidoreductase Activity. *Journal of Clinical Biochemistry and Nutrition*, *45*(3): 285–291.

Hak, A. E., Choi, H. K., (2008, March). Lifestyle and gout. *Current Opinion in Rheumatology*, *20*(2): 179–186.

Halliwell, B. (1996). Commentary: Vitamin C: Antioxidant or Pro-Oxidant In Vivo? *Free Radical Research*, *25*(5): 439–454.

Halliwell, B., & Gutteridge, J. M. (1990). Role of free radicals and catalytic metal ions in human disease: an overview. *Methods in Enzymology*, *186*: 1–85.

Hämäläinen, M., Nieminen, R., Vuorela, P., Heinonen, M., & Moilanen, E. (2007). Anti-Inflammatory Effects of Flavonoids: Genistein, Kaempferol, Quercetin, and Daidzein Inhibit STAT-1 and NF-κB Activations, Whereas Flavone, Isorhamnetin, Naringenin, and Pelargonidin Inhibit only NF-κB Activation along with Their Inhibitory Effect on iNOS Expression and NO Production in Activated Macrophages. *Mediators of Inflammation*, *45673*.

Hamburger, M. O., Cordell, G. A., & Ruangrungsi, N. (1991). Traditional medicinal plants of Thailand XVII Biologically active constituents of *Plumeria rubra*. *Journal of Ethnopharmacology*, *33*(3):289–292.

Han, J., Bang, M., Chun, O., Kim, D., Lee, C., & Baek, N. (2004). Flavonol glycosides from the aerial parts of *Aceriphyllum rossii* and their antioxidant activities. *Archives of Pharmacal Research*, *27*(4): 390–395.

Handa, S. S. (2008). An Overview of Extraction Techniques for Medicinal and Aromatic Plants In : Handa, S. S., Khanuja, S. P. S., Longo, G., and Rakesh D. D. (eds.) *Extraction Technologies for Medicinal and Aromatic Plants. International centre for Science and High Technology.*

Harborne, J. B. (1984). Methods of Plant Analysis. *Phytochemical Methods*: 1-36.

Harris, C. M., & Massey, V. (1997). The Oxidative Half-reaction of Xanthine Dehydrogenase with NAD; Reaction Kinetics and Steady-state Mechanism. *Journal of Biological Chemistry*, *272*(45): 28335–28341.

Harrison, R. (2002). Structure and function of xanthine oxidoreductase: where are we now?. *Free Radical Biology and Medicine*, *33*(6): 774–797.

Haskard, D. O., & Landis, R. C. (2002). Interactions between leukocytes and endothelial cells in gout: lessons from a self-limiting inflammatory response. *Arthritis Research*, *4*(Suppl 3): S91–S97.

Hayouni, E. A., Abedrabba, M., Bouix, M., & Hamdi, M. (2007). The effects of solvents and extraction method on the phenolic contents and biological activities *in vitro* of *Tunisian Quercus coccifera L.* and *Juniperus phoenicea* L. fruit extracts. *Food Chemistry*, *105*(3): 1126–1134.

Heijnen, C. G., Haenen, G. R., Vekemans, J. A., & Bast, A. (2001). Peroxynitrite scavenging of flavonoids: structure activity relationship. *Environmental Toxicology and Pharmacology*, *10*(4): 199–206.

Heinig, M., & Johnson, R. J. (2006). Role of uric acid in hypertension, renal disease, and metabolic syndrome. *Cleveland Clinic Journal of Medicine, 73*(12): 1059–1064.

Heo, S. J., Yoon, W. J., Kim, K. N., Ahn, G. N., Kang, S. M., Kang, D. H., ... & Jeon, Y. J. (2010). Evaluation of Anti-Inflammatory Effect of Fucoxanthin Isolated from Brown Algae in Lipopolysaccharide-Stimulated RAW 264.7 Macrophages. *Food and Chemical Toxicology*, *48*(8): 2045–2051.

Hoffman, H. M., Scott, P., Mueller, J. L., Misaghi, A., Stevens, S., Yancopoulos, G. D.,.. & Liu-Bryan, R. (2010). Role of the Leucine-Rich Repeat Domain of Cryopyrin/NALP3 in Monosodium Urate Crystal–Induced Inflammation in Mice. *Arthritis & Rheumatism*, *62*(7): 2170–2179.

Hoffstein, S., & Weissmann, G. (1975). Mechanisms of lysosomal enzyme release from leukocytes. *Arthritis & Rheumatism*, *18*(2): 153–165.

Hong, S., Joo, T., & Jhoo, J. W. (2015). Antioxidant and anti-inflammatory activities of 3, 5-dicaffeoylquinic acid isolated from *Ligularia fischeri* leaves. *Food Science and Biotechnology*, *24*(1): 257–263.

Hoskison, K. T., & Wortmann, R. L. (2007). Management of Gout in Older Adults. *Drugs & Aging*, *24*(1): 21–36.

Hossain, M. B., Patras, A., Barry-Ryan, C., Martin-Diana, A. B., & Brunton, N. P. (2011). Application of principal component and hierarchical cluster analysis to classify different spices based on *in vitro* antioxidant activity and individual polyphenolic antioxidant compounds. *Journal of Functional Foods*, *3*(3): 179–189.

Hou, C., Lee, Y., Hung, H., Fu, H., & Jeng, K. (2012). Longan Seed Extract Reduces Hyperuricemia via Modulating Urate Transporters and Suppressing Xanthine Oxidase Activity. *The American Journal of Chinese Medicine*, *40*(05): 979–991.

Houghton, P. J., & Osibogun, I. M. (1993). Flowering Plants Used Against Snakebite. *Journal of Ethnopharmacology*, *39*(1): 1–29.

Huang, J., Wang, S., Zhu, M., Chen, J., & Zhu, X. (2011). Effects of Genistein, Apigenin, Quercetin, Rutin and Astilbin on Serum Uric Acid Levels and Xanthine Oxidase Activities in Normal and Hyperuricemic Mice. *Food and Chemical Toxicology*, *49*(9): 1943–1947.

Huber, R., Hof, P., Duarte, R. O., Moura, J. J., Moura, I., Liu, M. Y., … Romão, M. J. (1996). A Structure-Based Catalytic Mechanism for the Xanthine Oxidase Family of Molybdenum Enzymes. *Proceedings of the National Academy of Sciences of the United States of America*, *93*(17): 8846–8851.

Hung, T. M., Na, M., Thuong, P. T., Su, N. D., Sok, D., Song, K. S., & Bae, K. (2006). Antioxidant activity of caffeoyl quinic acid derivatives from the roots of *Dipsacus asper* Wall. *Journal of Ethnopharmacology*, *108*(2): 188–192.

Hwang, Y. G., & Saag, K. G. (2013). Epidemiology of gout. *Gout* : 6–23.

Hyslop, P. A., Hinshaw, D. B., Halsey, W. A., Schraufstätter, I. U., Sauerheber, R. D., Spragg, R. G.,& Cochrane, C. (1988). Mechanisms of oxidant-mediated cell injury. The glycolytic and mitochondrial pathways of ADP phosphorylation are major intracellular targets inactivated by hydrogen peroxide. *Journal of Biological Chemistry*, *263*(4): 1665–1675.

Iwalewa, E., Iwalewa, O., & Adeboye, J. (2003). Analgesic, antipyretic, anti-inflammatory effects of methanol, chloroform and ether extracts of *Vernonia cinerea* less leaf. *Journal of Ethnopharmacology*, *86*(2-3): 229–234.

Iwasaki, T., Okamoto, K., Nishino, T., Mizushima, J., Hori, H., & Nishino, T. (2000). Sequence Motif-Specific Assignment of Two [2Fe-2S] Clusters in Rat Xanthine Oxidoreductase Studied by Site-Directed Mutagenesis. *Journal of Biochemistry*, *127*(5): 771–778.

Jiao, R. H., Ge, H. M., Shi, D. H., & Tan, R. X. (2006). An Apigenin-Derived Xanthine Oxidase Inhibitor from *Palhinhaea cernua*. *Journal of Natural Products*, *69*(7): 1089–1091.

Jia, Z., & Tian, C. (2009). Quantitative determination of polyethylene glycol with modified Dragendorff reagent method. *Desalination*, *247*(1-3): 423–429.

Kalemkerian, G. P., Darwish, B., & Varterasian, M. L. (1997). Tumor Lysis Syndrome in Small Cell Carcinoma and Other Solid Tumors. *The American Journal of Medicine*, *103*(5): 363–367.

Kanevets, U., Sharma, K., Dresser, K., & Shi, Y. (2009). A Role of IgM Antibodies in Monosodium Urate Crystal Formation and Associated Adjuvanticity. *The Journal of Immunology*, *182*(4): 1912–1918.

Keenan, R. T., O'Brien, W. R., Lee, K. H., Crittenden, D. B., Fisher, M. C., Goldfarb, D. S., & Pillinger, M. H. (2011). Prevalence of contraindications and prescription of pharmacologic therapies for gout. *The American Journal of Medicine*, *124*(2): 155–163.

Kelso, J. M., Keating, R. M. (1996, May). Successful desensitization for treatment of a fixed drug eruption to allopurinol. *Journal of Allergy and Clinical Immunology*, *97*(5): 1171–1172.

Khaliq, A., Sabir, S. M., Ahmed, S. D., Boligon, A. A., Athayde, M. L., Jabbar, A. J., & Khan, A. (2015). Antioxidant activities and phenolic composition of Olive *(Olea europaea)* leaves. *Journal of Applied Botany and Food Quality*, *88*(1): 16–21.

Khanna, D., Fitzgerald, J. D., Khanna, P. P., Bae, S., Singh, M. K., Neogi, T., . . . Terkeltaub, R. (2012). 2012 American College of Rheumatology guidelines for management of gout. Part 1: Systematic nonpharmacologic and pharmacologic therapeutic approaches to hyperuricemia. *Arthritis Care & Research*, *64*(10): 1431–1446.

Khanna, D., Khanna, P. P., Fitzgerald, J. D., Singh, M. K., Bae, S., Neogi, T., ... & Kaldas, M. (2012). 2012 American College of Rheumatology guidelines for management of gout. Part 2: Therapy and antiinflammatory prophylaxis of acute gouty arthritis. *Arthritis Care & Research*, *64*(10): 1447–1461.

Kim, J. H., Ryan, M. G., Knaut, H., & Hille, R. (1996). The Reductive Half-reaction of Xanthine Oxidase the Involvement of Prototrophic Equilibra in The Course of The Catalytic Sequence. *Journal of Biological Chemistry*, *271*(12): 6771–6780.

Kim, K. Y., Schumacher, H. R., Hunsche, E., Wertheimer, A. I., & Kong, S. X. (2003). A literature review of the epidemiology and treatment of acute gout. *Clinical Therapeutics*, *25*(6): 1593–1617.

Kisker, C., Schindelin, H., Baas, D., Rétey, J., Meckenstock, R. U., & Kroneck, P. M. (1998). A Structural Comparison of Molybdenum Cofactor-Containing Enzymes. *FEMS Microbiology Reviews*, *22*(5): 503–521.

Kobayashi, Y. (2010). The regulatory role of nitric oxide in proinflammatory cytokine expression during the induction and resolution of inflammation. *Journal of Leukocyte Biology*, *88*(6): 1157–1162.

Kong, L. D., Cai, Y., Huang, W. W., Cheng, C. H., & Tan, R. X. (2000). Inhibition of Xanthine Oxidase by Some Chinese Medicinal Plants Used To Treat Gout. *Journal of Ethnopharmacology*, *73*(1): 199–207.

Kong, L. D., Yang, C., Ge, F., Wang, H. D., & Guo, Y. S. (2004). A Chinese Herbal Medicine Ermiao Wan Reduces Serum Uric Acid Level and Inhibits Liver Xanthine Dehydrogenase and Xanthine Oxidase in Mice. *Journal of Ethnopharmacology*, *93*(2): 325–330.

Kong, L. D., Zhang, Y., Pan, X., Tan, R. X., & Cheng, C. H. K. (2000). Inhibition of Xanthine Oxidase by Liquiritigenin and Isoliquiritigenin Isolated from *Sinofranchetia chinensis*. *Cellular and Molecular Life Sciences CMLS*, *57*(3): 500–505.

Krenitsky, T. A., Spector, T., & Hall, W. W. (1986). Xanthine oxidase from human liver: purification and characterization. *Archives of biochemistry and biophysics*, *247*(1): 108–119.

Kulkarni, A. P., Aradhya, S. M., & Divakar, S. (2004). Isolation and identification of a radical scavenging antioxidant– punicalagin from pith and carpellary membrane of pomegranate fruit. *Food Chemistry, 87*(4): 551–557.

Kumar, A., Ilavarasan, R., Jayachandran, T., Decaraman, M., Aravindhan, P., Padmanabhan, N., & Krishnan, M. R. V. (2009). Phytochemicals investigation on a tropical plant, *Syzygium cumini* from Kattuppalayam, Erode district, Tamil Nadu, South India. *Pakistan Journal of Nutrition, 8*(1): 83–85.

Kumar, U. S., Tiwari, A. K., Reddy, S. V., Aparna, P., Rao, R. J., Ali, A. Z., & Rao, J. M. (2005). Free-Radical-Scavenging and Xanthine Oxidase Inhibitory Constituents from *Stereospermum personatum*. *Journal of Natural Products*, *68*(11): 1615–1621.

Lima, R. D., Ferrari, F. C., Souza, M. R., Pereira, B. M., Paula, C. A., & Saúde-Guimarães, D. A. (2015). Effects of extracts of leaves from *Sparattosperma leucanthum* on hyperuricemia and gouty arthritis. *Journal of Ethnopharmacology*, *161*: 194–199.

Lim, T. K. (2011). *Edible medicinal and non-medicinal plants*. Dordrecht: Springer.

Liu, L., Cheng, S., Shieh, P., Lee, J., Chen, J., Ho, C., . . . Way, T. (2014). The methanol extract of *Euonymus laxiflorus*, *Rubia lanceolata* and *Gardenia jasminoides* inhibits xanthine oxidase and reduce serum uric acid level in rats. *Food and Chemical Toxicology*, *70*: 179–184.

Liu, X., Chen, R., Shang, Y., Jiao, B., & Huang, C. (2008). Lithospermic acid as a novel xanthine oxidase inhibitor has anti-inflammatory and hypouricemic effects in rats. *Chemico-Biological Interactions*, *176*(2-3): 137–142.

Lue, B. M., Nielsen, N. S., Jacobsen, C., Hellgren, L., Guo, Z., & Xu, X. (2010). Antioxidant properties of modified rutin esters by DPPH, reducing power, iron chelation and human low density lipoprotein assays. *Food Chemistry*, *123*(2): 221–230.

Luzia, M., Trugo, L., Paixão, K. D., Marcílio, R., Maria, C. D., & Quinteiro, L. (1998). Effect of 5-Caffeoylquinic Acid in the Presence of Metal Chelators on Soybean Oil Oxidative Stability. *LWT - Food Science and Technology*, *31*(1): 64–68.

Ma, X., Li, H., Dong, J., & Qian, W. (2011). Determination of hydrogen peroxide scavenging activity of phenolic acids by employing gold nanoshells precursor composites as nanoprobes. *Food Chemistry*, *126*(2): 698–704.

MacDonald-Wicks, L. K., Wood, L. G., & Garg, M. L. (2006). Methodology for the determination of biological antioxidant capacity *in vitro*: a review. *Journal of the Science of Food and Agriculture, 86*(13): 2046–2056.

MacNee, W. (2001). Oxidative stress and lung inflammation in airways disease. *European Journal of Pharmacology*, *429*(1): 195–207.

Madhavi, D. L., Deshpande, S. S., & Salunkhe, D. K. (1996). *Food antioxidants: technological, toxicological, and health perspectives.* New York: Marcel Dekker.

Mandell, B. F. (2002). Hyperuricemia and gout: a reign of complacency. *Cleveland Clinic Journal of Medicine*, *69*(8): 589–590.

Mărghitaş, L. A., Stanciu, O. G., Dezmirean, D. S., Bobiş, O., Popescu, O., Bogdanov, S., & Campos, M. G. (2009). *In vitro* antioxidant capacity of honeybee-collected pollen of selected floral origin harvested from Romania. *Food Chemistry*, *115*(3): 878–883.

Marson, P., & Pasero, G. (2011). Some historical remarks on microcrystalline arthritis (gout and chondrocalcinosis). *Reumatismo*, *63*(4): 199–206.

Martínez-Flórez, S., Gutiérrez-Fernández, B., Sánchez-Campos, S., González-Gallego, J., & Tuñón, M. J. (2005). Quercetin attenuates nuclear factor-κB activation and nitric oxide production in interleukin-1β–activated rat hepatocytes. *The Journal of Nutrition*, *135*(6): 1359–1365.

Martinon, F., Pétrilli, V., Mayor, A., Tardivel, A., & Tschopp, J. (2006). Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature*, *440*(7081): 237–241.

Masuoka, N., Matsuda, M., & Kubo, I. (2012). Characterisation of the antioxidant activity of flavonoids. *Food Chemistry*, *131*(2): 541–545.

Mathew, B., Suresh, J., E Mathew, G., A Rasheed, S., K Vilapurathu, J., & Jayaraj, P. (2015). Flavonoids: An Outstanding Structural Core for the Inhibition of Xanthine Oxidase Enzyme. *Current Enzyme Inhibition, 11*(2):108–115.

McMaster, J., & Enemark, J. H. (1998). The Active Sites of Molybdenum-and Tungsten-Containing Enzymes. *Current Opinion in Chemical Biology*, *2*(2): 201–207.

McNearney, T., Baethge, B. A., Cao, S., Alam, R., Lisse, J. R., & Westlund, K. N. (2004). Excitatory amino acids, TNF-α, and chemokine levels in synovial fluids of patients with active arthropathies. *Clinical & Experimental Immunology, 137*(3): 621–627.

Medini, F., Fellah, H., Ksouri, R., & Abdelly, C. (2014). Total phenolic, flavonoid and tannin contents and antioxidant and antimicrobial activities of organic extracts of shoots of the plant *Limonium delicatulum*. *Journal of Taibah University for Science*, *8*(3): 216–224.

Melidou, M., Riganakos, K., & Galaris, D. (2005). Protection against nuclear DNA damage offered by flavonoids in cells exposed to hydrogen peroxide: the role of iron chelation. *Free Radical Biology and Medicine, 39*(12): 1591–1600.

Meng, Z., Tang, Z., Yan, Y., Guo, C., Cao, L., Ding, G., . . . Yang, Z. (2014). Study on the Anti-Gout Activity of Chlorogenic Acid: Improvement on Hyperuricemia and Gouty Inflammation. *The American Journal of Chinese Medicine*, *42*(06): 1471–1483.

Merina, A. J., Sivanesan, D., Begum, V. H., & Sulochana, N. (2010). Antioxidant and Hypolipidemic Effect of *Plumeria Rubra L.* in Alloxan Induced Hyperglycemic Rats. *E-Journal of Chemistry*, *7*(1): 1–5.

Merriman, T. R., & Dalbeth, N. (2011). The genetic basis of hyperuricaemia and gout. J*oint Bone Spine, 78*(1): 35–40.

Miao, Z., Li, C., Chen, Y., Zhao, S., Wang, Y., Wang, Z., & Wang, C. Y. (2008). Dietary and lifestyle changes associated with high prevalence of hyperuricemia and gout in the Shandong coastal cities of Eastern China. *The Journal of Rheumatology*, *35*(9): 1859–1864.

Michet, C. J., Evans, J. M., Fleming, K. C., O'duffy, J. D., Jurisson, M. L., & Hunder, G. G. (1995). Common Rheumatologic Diseases in Elderly Patients. *Mayo Clinic Proceedings*, *70*(12): 1205–1214.

Mikuls, T. R. (2005). Gout epidemiology: results from the UK General Practice Research Database, 1990-1999. *Annals of the Rheumatic Diseases*, *64*(2): 267–272.

Mo, S. F., Zhou, F., Lv, Y. Z., Hu, Q. H., Zhang, D. M., & Kong, L. D. (2007). Hypouricemic action of selected flavonoids in mice: structure-activity relationships. *Biological and Pharmaceutical Bulletin*, *30*(8): 1551–1556.

Mondal, Madhu Sudan, Apurba Kumar Sau, and Samaresh Mitra. (2000) "Mechanism of the inhibition of milk xanthine oxidase activity by metal ions: a transient kinetic study." *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology 1480* (1-2) : 302– 310.

.

Mondal, M. S., & Mitra, S. (1996). The Inhibition of Bovine Xanthine Oxidase Activity by Hg^{2+} and Other Metal Ions. *Journal of Inorganic Biochemistry, 62* (4) : 271–279.

Montoro, P., Braca, A., Pizza, C., & De Tommasi, N. (2005). Structure–antioxidant activity relationships of flavonoids isolated from different plant species. *Food chemistry*, *92*(2): 349–355.

Monzon, R. B. (1995). Traditional medicine in the treatment of parasitic diseases in the Philippines. *Southeast Asian Journal of Tropical Medicine and Public Health*, *26*(3): 421–428.

Müller, L., Gnoyke, S., Popken, A. M., & Böhm, V. (2010). Antioxidant capacity and related parameters of different fruit formulations. *LWT-Food Science and Technology*, *43*(6): 992–999.

Murugaiyah, V., & Chan, K. L. (2009). Mechanisms of Antihyperuricemic Effect of *Phyllanthus niruri* and Its Lignan Constituents. *Journal of Ethnopharmacology*, *124*(2): 233–239.

Nagao, A., Seki, M., & Kobayashi, H. (1999). Inhibition of xanthine oxidase by flavonoids. *Bioscience, Biotechnology, and Biochemistry*, *63*(10): 1787–1790.

Nair, M. P., Mahajan, S., Reynolds, J. L., Aalinkeel, R., Nair, H., Schwartz, S. A., & Kandaswami, C. (2006). The flavonoid quercetin inhibits proinflammatory cytokine (tumor necrosis factor alpha) gene expression in normal peripheral blood mononuclear cells via modulation of the NF-κβ system. *Clinical and Vaccine Immunology*, *13*(3): 319–328.

Nakagawa, T., Tuttle, K. R., Short, R. A., & Johnson, R. J. (2005). Hypothesis: fructose-induced hyperuricemia as a causal mechanism for the epidemic of the metabolic syndrome. *Nature Clinical Practice Nephrology*, *1*(2): 80–86.

Nakai, K., Kadiiska, M. B., Jiang, J. J., Stadler, K., & Mason, R. P. (2006). Free radical production requires both inducible nitric oxide synthase and xanthine oxidase in LPS-treated skin. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(12): 4616–4621.

Namiki, M. (1990). Antioxidants/antimutagens in food. *Critical Reviews in Food Science & Nutrition, 29*(4): 273–300.

Nargis, A., Abdul, M., Ali, S. N., Kazmi, S.U. (1994). Rubrinol. A New Antibacterial Triterpenoid from *Plumeria rubra*. *Fitoterapi*a, *65*(2): 162–166.

Neogi, T. (2011). Gout. *New England Journal of Medicine*, *364*(5): 443–452

Newcombe, D. S. (1975). *Inherited biochemical disorders and uric acid metabolism*. Baltimore: University Park Press.

Nguyen, Mai Thanh Thi, Suresh Awale, Yasuhiro Tezuka, Quan Le Tran, Hiroshi Watanabe, and Shigetoshi Kadota (2004). Xanthine Oxidase Inhibitory Activity of Vietnamese Medicinal Plants.*Biological & Pharmaceutical Bulletin 27*(9):1414–421.

Nishino, T. (1994). The conversion of xanthine dehydrogenase to xanthine oxidase and the role of the enzyme in reperfusion injury. *Journal of Biochemistry*, *116*(1): 1–6.

Nishino, T., & Okamoto, K. (2015). Mechanistic insights into xanthine oxidoreductase from development studies of candidate drugs to treat hyperuricemia and gout. *Journal of Biological Inorganic Chemistry*, *20*(2): 195–207.

Nuki, G. (2006). Gout. *Medicine, 34*(10): 417–423.

Nuki, G., & Simkin, P. A. (2006). A concise history of gout and hyperuricemia and their treatment. *Arthritis Research & Therapy*, *8*(Suppl 1): S1.

Okamoto, K., Kusano, T., & Nishino, T. (2013). Chemical Nature and Reaction Mechanisms of the Molybdenum Cofactor of Xanthine Oxidoreductase. *Current Pharmaceutical Design*, *19*(14): 2606–2614.

Okamoto, K., Matsumoto, K., Hille, R., Eger, B. T., Pai, E. F., & Nishino, T. (2004). The crystal structure of xanthine oxidoreductase during catalysis: implications for reaction mechanism and enzyme inhibition. *Proceedings of the National Academy of Sciences of the United States of America, 101*(21): 7931–7936.

Oliviero, F., Scanu, A., & Punzi, L. (2011). Metabolism of crystals within the joint. *Reumatismo*, *63*(4): 221–229.

Ordonez, A. A. L., Gomez, J. D., Vattuone, M. A., & Isla, M. I. (2006). Antioxidant activities of *Sechium edule* (Jacq.) Swartz extracts. *Food Chemistry*, *97*(3): 452–458.

Owen, P. L., & Johns, T. (1999). Xanthine Oxidase Inhibitory Activity of Northeastern North American Plant Remedies used for Gout. *Journal of Ethnopharmacology*, *64*(2): 149–160.

Pacher, P. (2006). Therapeutic Effects of Xanthine Oxidase Inhibitors: Renaissance Half a Century after the Discovery of Allopurinol. *Pharmacological Reviews*, *58*(1): 87–114.

Palu, A., Deng, S., West, B., & Jensen, J. (2009). Xanthine oxidase inhibiting effects of noni (*Morinda citrifolia*) fruit juice. *Phytotherapy Research*, *23*(12): 1790–1791.

Pannala, A. S., Chan, T. S., O'brien, P. J., & Rice-Evans, C. A. (2001). Flavonoid B-Ring Chemistry and Antioxidant Activity: Fast Reaction Kinetics. *Biochemical and Biophysical Research Communications*, *282*(5): 1161–1168.

Parks, D. A., & Granger, D. N. (1986). Contributions of ischemia and reperfusion to mucosal lesion formation. *American Journal of Physiology–Gastrointestinal and Liver Physiology*, *250*(6): G749–G753.

Patton, S., and Keenan, T. W. (1975).The milk fat globule membrane. *Biochim. Biophys. Acta*, *415*(3): 273–309.

Pereira, D. M., Faria, J., Gaspar, L., Ferreres, F., Valentão, P., Sottomayor, M., & Andrade, P. B. (2010). Exploiting *Catharanthus roseus* roots: Source of antioxidants. *Food Chemistry*, *121*(1): 56–61.

Piccini, A., Carta, S., Tassi, S., Lasiglié, D., Fossati, G., & Rubartelli, A. (2008). ATP is released by monocytes stimulated with pathogen-sensing receptor ligands and induces IL-1β and IL-18 secretion in an autocrine way. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(23): 8067–8072.

Pino, J. A., Ferrer, A., Alvarez, D., & Rosado, A. (1994). Volatiles of an alcoholic extract of flowers from *Plumeria rubra* L. var. *acutifolia*. *Flavour and fragrance Journal*, *9*(6): 343–345.

Primatesta, P., Plana, E., & Rothenbacher, D. (2011). Gout treatment and comorbidities: a retrospective cohort study in a large US managed care population. *BMC Musculoskeletal Disorders, 12*(1).

Procházková, D., Boušová, I., & Wilhelmová, N. (2011). Antioxidant and prooxidant properties of flavonoids. *Fitoterapia*, *82*(4): 513–523.

Pu, W., Wang, D., & Zhou, D. (2015). Structural Characterization and Evaluation of the Antioxidant Activity of Phenolic Compounds from *Astragalus taipaishanensis* and Their Structure–Activity Relationship. *Scientific Reports*, *5*: 13914.

Puig, J. G., Antón, F. M., Jiménez, M. L., & Gutiérrez, P. C. (1986). Renal handling of uric acid in gout: Impaired tubular transport of urate not dependent on serum urate levels. *Metabolism*, *35*(12): 1147–1153.

Punzi, L., Podswiadek, M., Sfriso, P., Oliviero, F., Fiocco, U., & Todesco, S. (2007). Pathogenetic and clinical rationale for TNF-blocking therapy in psoriatic arthritis. *Autoimmunity reviews*, *6*(8): 524–528.

Punzi, L., Scanu, A., Ramonda, R., & Oliviero, F. (2012). Gout as autoinflammatory disease: new mechanisms for more appropriated treatment targets. *Autoimmunity reviews*, *12*(1): 66–71.

Rahman, I., Gilmour, P. S., Jimenez, L. A., & Macnee, W. (2002). Oxidative stress and TNF-a induce histone Acetylation and NF-κB/AP-1 activation in Alveolar epithelial cells: Potential mechanism In gene transcription in lung inflammation. Oxygen/Nitrogen Radicals: *Cell Injury and Disease*: 239–248.

Rangan, G. K., Wang, Y., Tay, Y. C., & Harris, D. C. (1999). Inhibition of NFκB activation with antioxidants is correlated with reduced cytokine transcription in PTC. *American Journal of Physiology-Renal Physiolog*y, *277*(5): F779–F789.

Ramonda, R., Nigro, A. L., Modesti, V., Nalotto, L., Musacchio, E., Iaccarino, L., & Doria, A. (2011). Atherosclerosis in psoriatic arthritis. *Autoimmunity reviews*, *10*(12): 773–778.

Rasaratnam, I., & Christophidis, N. (1995). Gout:'a disease of plenty'. *Australian family physician, 24*(5): 849–851.

Rashidi, M. R., Haidari, F., Keshavarz, S. A., Mahboob, S. A., Eshraghian, M. R., & Shahi, M. M. (2008). Effects of Onion on Serum Uric Acid Levels and Hepatic Xanthine Dehydrogenase/Xanthine Oxidase Activities in Hyperuricemic Rats. *Pakistan Journal of Biological Sciences*, *11*(14): 1779–1784.

Ratnam, K. (1995). The Molybdenum Centers of Xanthine Oxidase and Xanthine Dehydrogenase. *Journal of Biological Chemistry*, *270*(33): 19209–19212.

Reinders, M. K., Van Roon, E. N., Jansen, T. T. A., Delsing, J., Griep, E. N., Hoekstra, M., & Brouwers, J. R. B. J. (2009). Efficacy and tolerability of urate-lowering drugs in gout: a randomised controlled trial of benzbromarone versus probenecid after failure of allopurinol. *Annals of the rheumatic diseases*, *68*(1): 51–56.

Richette, P., & Bardin, T. (2010). Colchicine for the treatment of gout. *Expert Opinion on Pharmacotherapy*, *11*(17): 2933–2938.

Robinson, T. (2012). *The biochemistry of alkaloids (Vol. 3)*. Springer Science & Business Media.

Roddy, E., & Doherty, M. (2010). Gout. Epidemiology of gout. *Arthritis Research & Therapy*, *12*(6): 223.

Roddy, E., Zhang, W., & Doherty, M. (2007). The changing epidemiology of gout. *Nature Clinical Practice Rheumatology, 3*(8): 443–449.

Rodrigues, N. P., Benassi, M. T., & Bragagnolo, N. (2014). Scavenging capacity of coffee brews against oxygen and nitrogen reactive species and the correlation with bioactive compounds by multivariate analysis. *Food Research International*, *61*: 228–235.

Rohman, A., Riyanto, S., Yuniarti, N., Saputra, W. R., Utami, R., & Mulatsih, W. (2010). Antioxidant activity, total phenolic and total flavonoid of extracts and fractions of red fruit (*Pandamus conoideus* Lam). *International Food Research Journal, 17*: 97–106.

Royer, M., Diouf, P. N., & Stevanovic, T. (2011). Polyphenol contents and radical scavenging capacities of red maple (*Acer rubrum* L.) extracts. *Food and Chemical Toxicology*, *49*(9): 2180–2188.

Ruilope, L. M., & Garcia-Puig, J. (2001). Hyperuricemia and renal function. *Current Hypertension Reports*, *3*(3): 197–202.

Sabina, E. P., Nagar, S., & Rasool, M. (2010). A Role of Piperine on Monosodium Urate Crystal-Induced Inflammation—An Experimental Model of Gouty Arthritis. *Inflammation*, *34*(3): 184–192.

Sahreen, S., Khan, M. R., & Khan, R. A. (2010). Evaluation of antioxidant activities of various solvent extracts of *Carissa opaca* fruits. *Food chemistry*, *122*(4): 1205–1211.

Salazar, R., Pozos, M. E., Cordero, P., Perez, J., Salinas, M. C., & Waksman, N. (2008). Determination of the Antioxidant Activity of Plants from Northeast Mexico. *Pharmaceutical Biology*, *46*(3): 166–170.

Salem, J. H., Chevalot, I., Harscoat-Schiavo, C., Paris, C., Fick, M., & Humeau, C. (2011). Biological activities of flavonoids from *Nitraria retusa* (Forssk.) Asch. and their acylated derivatives. *Food Chemistry*, *124*(2): 486–494.

Sarnesto, A., Linder, N., & Raivio, K. O. (1996). Organ distribution and molecular forms of human xanthine dehydrogenase/xanthine oxidase protein. *Laboratory Investigation; A Journal of Technical Methods and Pathology*, *74*(1): 48–56.

Scanu, A., Oliviero, F., Ramonda, R., Frallonardo, P., Dayer, J., & Punzi, L. (2012). Cytokine levels in human synovial fluid during the different stages of acute gout: role of transforming growth factor β1 in the resolution phase. *Annals of the Rheumatic Diseases*, *71*(4): 621–624.

Schröder, K., Vecchione, C., Jung, O., Schreiber, J. G., Shiri-Sverdlov, R., van Gorp, P. J., & Brandes, R. P. (2006). Xanthine oxidase inhibitor tungsten prevents the development of atherosclerosis in ApoE knockout mice fed a Western-type diet. *Free Radical Biology and Medicine, 41*(9): 1353–1360.

Scott, P., Ma, H., Viriyakosol, S., Terkeltaub, R., & Liu-Bryan, R. (2006). Engagement of CD14 Mediates the Inflammatory Potential of Monosodium Urate Crystals. *The Journal of Immunology*, *177*(9): 6370–6378.

Seabra, R. M., Andrade, P. B., Valentão, P., Fernandes, E., Carvalho, F., & Bastos, M. L.(2006). Anti-oxidant compounds extracted from several plant materials. In M. Fingerman, & R. Nagabhushanam (Eds.), *Biomaterials from aquatic and terrestrial organisms* (pp. 115−174). Jersey Plymouth, USA: New Hampshire: Science Publishers – Enfield (NH).

Seegmiller, J. E., Laster, L., & Howell, R. R. (1963). Biochemistry of Uric Acid and Its Relation to Gout. *New England Journal of Medicine*, *268*(14): 764–773.

Selloum, L., Reichl, S., Müller, M., Sebihi, L., & Arnhold, J. (2001). Effects of Flavonols on the Generation of Superoxide Anion Radicals by Xanthine Oxidase and Stimulated Neutrophils. *Archives of Biochemistry and Biophysics*, *395*(1): 49–56.

Sestili, P., Guidarelli, A., Dachà, M., & Cantoni, O. (1998). Quercetin prevents DNA single strand breakage and cytotoxicity caused by tert-butylhydroperoxide: free radical scavenging versus iron chelating mechanism. *Free Radical Biology and Medicine*, *25*(2): 196–200.

Seyoum, A., Asres, K., El-Fiky, F.K. (2006). Structure-radical scavenging activity relationships of flavonoids. *Phytochemistry*, *67*(18): 2058–2070.

Shahidi, F., Wanasundara, U. N., & Amarowicz, R. (1994). Natural antioxidants from low-pungency mustard flour. *Food Research International*, *27*(5): 489–493.

Sharma, D., Rawat, I., & Goel, H. C. (2012). Antioxidant and prebiotic potential of some cucurbits. *Research Journal of Medicinal Plant*, *6*(7): 500–510.

Shi, J., Gong, J., Liu, J. E., Wu, X., & Zhang, Y. (2009). Antioxidant capacity of extract from edible flowers of *Prunus mume* in China and its active components. *LWT-Food Science and Technology*, *42*(2): 477–482.

Shi, Y. W., Wang, C. P., Wang, X., Zhang, Y. L., Liu, L., Wang, R. W., & Kong, L. D. (2012). Uricosuric and Nephroprotective Properties of *Ramulus Mori* Ethanol Extract in Hyperuricemic Mice. *Journal of Ethnopharmacology*, *143*(3): 896–904.

Shoji, A., Yamanaka, H., & Kamatani, N. (2004). A retrospective study of the relationship between serum urate level and recurrent attacks of gouty arthritis: Evidence for reduction of recurrent gouty arthritis with antihyperuricemic therapy. *Arthritis Care & Research*, *51*(3): 321–325.

Shukla, S., Mehta, A., Mehta, P., & Bajpai, V. K. (2012). Antioxidant ability and total phenolic content of aqueous leaf extract of *Stevia rebaudiana* Bert. *Experimental and Toxicologic Pathology*, *64*(7-8): 807–811.

Silva, C. R., Fröhlich, J. K., Oliveira, S. M., Cabreira, T. N., Rossato, M. F., Trevisan, G., & Ferreira, J. (2013). The Antinociceptive and Anti-Inflammatory Effects of The Crude Extract of *Jatropha isabellei* In a Rat Gout Model. *Journal of Ethnopharmacology*, *145*(1): 205–213.

Singh, J. A., Reddy, S. G., & Kundukulam, J. (2011). Risk Factors for Gout and Prevention: A Systematic Review of the Literature. *Current Opinion in Rheumatology*, *23*(2): 192–202.

Sintayehu, B. (2012). Radical scavenging activities of the leaf extracts and a flavonoid glycoside isolated from *Cineraria abyssinica* Sch. Bip. Exa. Rich. *Journal of Applied Pharmaceutical Science*: 44–49.

Soung, D. Y., Rhee, S. H., Kim, J. S., Lee, J. Y., Yang, H. S., Choi, J. S., & Chung, H. Y. (2003). Peroxynitrite Scavenging Activity of Lithospermate B from *Salvia miltiorrhiza*. *Journal of Pharmacy and Pharmacology*, *55*(10): 1427–1432.

Spieker, L. E., Ruschitzka, F. T., Lüscher, T. F., & Noll, G. (2002). The management of hyperuricemia and gout in patients with heart failure. *European Journal of Heart Failure*, *4*(4): 403–410.

Srivastava, P., Raut, H. N., Wagh, R. S., Puntambekar, H. M., & Kulkarni, M. J. (2012). Purification and Characterization of An Antioxidant Protein ($\sim$ 16kDa) from *Terminalia chebula* fruit. *Food Chemistry*, *131*(1): 141–148.

Sroka, Z., & Cisowski, W. (2003). Hydrogen Peroxide Scavenging, Antioxidant and Anti-radical Activity of Some Phenolic Acids. *Food and Chemical Toxicology*, *41*(6): 753–758.

Stief, T. W. (2003). The physiology and pharmacology of singlet oxygen. *Medical Hypotheses, 60*(4): 567–572.

Stinefelt, B., Leonard, S. S., Blemings, K. P., Shi, X., & Klandorf, H. (2005). Free radical scavenging, DNA protection, and inhibition of lipid peroxidation mediated by uric acid. *Annals of Clinical & Laboratory Science, 35*(1): 37–45.

Stone, T. W., & Simmonds, H. A. (1991). *Purines: basic and clinical aspects*. Dordrecht: Kluwer Academic.

Sun, C., Fu, J., Chen, J., Jiang, L., Pan, Y. (2010). On-line HPLC method for screening of antioxidants against superoxide anion radical from complex mixtures. *Journal of Separation Science, 33*(8): 1018–1023.

Sundy, J. S. (2008). Gout management: Let's get it right this time. *Arthritis & Rheumatism*, *59*(11): 1535–1537.

Sweeney, A. P., Wyllie, S. G., Shalliker, R. A., & Markham, J. L. (2001). Xanthine Oxidase Inhibitory Activity of Selected Australian Native Plants. *Journal of Ethnopharmacology*, *75*(2): 273–277.

Szekanecz, Z., & Koch, A. E. (2007). Mechanisms of disease: angiogenesis in inflammatory diseases. *Nature Clinical Practice Rheumatology*, *3*(11): 635–643.

Taniguchi, A., Urano, W., Yamanaka, M., Yamanaka, H., Hosoyamada, M., Endou, H., & Kamatani, N. (2005). A common mutation in an organic anion transporter gene, SLC22A12, is a suppressing factor for the development of gout. *Arthritis & Rheumatism*, *52*(8): 2576–2577.

Teklehaymanot, T., Giday, M. (2007). Ethnobotanical Study of Medicinal Plants Used by People in Zegie Peninsula, Northwestern Ethiopia. *Journal of Ethnobiology and Ethnomedicine*, *3*(12): 1–11.

Teng, G. G., Nair, R., & Saag, K. G. (2006). Pathophysiology, Clinical Presentation and Treatment of Gout. *Drugs, 66*(12): 1547–1563.

Terkeltaub, R., Sundy, J. S., Schumacher, H. R., Murphy, F., Bookbinder, S., Biedermann, S. (2009). The interleukin 1 inhibitor rilonacept in treatment of chronic gout y arthritis: results of a placebo-controlled, monosequence crossover, non-randomised, single-blind pilot study. *Annals of Rheumatic Disease*, *68*(10): 1613–1617.

Theoduloz, C., Franco, L., Ferro, E., Hirschmann, G. S. (1988) Xanthine Oxidase Inhibitory Activity of Paraguayan *Myrtaceae*. *Journal of Ethnopharmacology*. *24*(2–3): 179–183.

Tohar, N., Awang, K., Mohd, M. A., & Jantan, I. (2006). Chemical composition of the essential oils of four *Plumeria* species grown on Peninsular Malaysia. *Journal of Essential Oil Research*, *18*(6): 613–617.

Tohar, N., Mohd, M. A., Jantan, I., & Awang, K. (2006). A comparative study of the essential oils of the genus *Plumeria* Linn. from Malaysia. *Flavour and Fragrance Journal*, *21*(6): 859–863.

Tretter, L., & Adam-Vizi, V. (2000). Inhibition of Krebs cycle enzymes by hydrogen peroxide: a key role of α-ketoglutarate dehydrogenase in limiting NADH production under oxidative stress. *The Journal of Neuroscience*, *20*(24): 8972–8979.

Tung, Y., & Chang, S. (2010). Inhibition of Xanthine Oxidase by *Acacia confusa* Extracts and Their Phytochemicals. *Journal of Agricultural and Food Chemistry*, *58*(2): 781–786.

Turkmen, N., Sari, F., & Velioglu, Y. S. (2006). Effects of extraction solvents on concentration and antioxidant activity of black and black mate tea polyphenols determined by ferrous tartrate and Folin–Ciocalteu methods. *Food Chemistry*, *99*(4): 835–841.

Umamaheswari, M., Asok Kumar, K., Somasundaram, A., Sivashanmugam, T., Subhadradevi, V. and Ravi, T. K. (2007). Xanthine Oxidase Inhibitory Activity of Some Indian Medicinal Plants. *Journal of Ethnopharmacology 109*(3): 547–551.

Urano W., Yamanaka H., Tsutani H., Nakajima H., Matsuda Y., Taniguchi A., Hara M., Kamatani N., (2002).The inflammatory process in the mechanism of decreased serum uric acid concentrations during acute gouty arthritis. *Journal of Rheumatology*, *29*(9): 1950–1953.

Vanacker, S., Tromp, M., Haenen, G., Vandervijgh, W., & Bast, A. (1995). Flavonoids as Scavengers of Nitric Oxide Radical. *Biochemical and Biophysical Research Communications*, *214*(3): 755–759.

Vázquez-Mellado, J., Alvarez Hernández, E., & Burgos-Vargas, R. (2004). Primary prevention in rheumatology: the importance of hyperuricemia. *Best Practice & Research Clinical Rheumatology, 18*(2): 111–124.

Vitart, V., Rudan, I., Hayward, C., Gray, N. K., Floyd, J., Palmer, C. N., . . . Wright, A. F. (2008). SLC2A9 is a newly identified urate transporter influencing serum urate concentration, urate excretion and gout. *Nature Genetics, 40*(4): 437–442.

Vivot, E., Muñoz, J. D., Cruañes, M. D., Cruañes, M. J., Tapia, A., Hirschmann, G. S., . . . Zacchino, S. (2001). Inhibitory activity of xanthine-oxidase and superoxide scavenger properties of Inga verna subsp. affinis. Its morphological and micrographic characteristics. *Journal of Ethnopharmacology*, *76*(1): 65–71.

Wagner, H., & Bladt, S. (2001). *Plant drug analysis: a thin layer chromatography atlas.* Berlin: Springer.

Wallace, K. L., Riedel, A. A., Joseph-Ridge, N., Wortmann, R. (2004) Increasing prevalence of gout and hyperuricemia over 10 years among older adults in a managed care population.*The Journal of Rheumatolology, 31*(8): 1582–1587.

Wanasundara, J. P., & Shahidi, F. (1994). Alkanol-ammonia-water/hexane extraction of flaxseed. *Food Chemistry*, *49*(1): 39–44.

Wang, C. C., Chang, S. C., Inbaraj, B. S., & Chen, B. H. (2010). Isolation of carotenoids, flavonoids and polysaccharides from Lycium barbarum L. and evaluation of antioxidant activity. *Food Chemistry*, *120*(1): 184–192.

Wang, J., Lou, J., Luo, C., Zhou, L., Wang, M., & Wang, L. (2012). Phenolic Compounds from *Halimodendron halodendron* (Pall.) Voss and Their Antimicrobial and Antioxidant Activities. *International Journal of Molecular Sciences*, *13*(12): 11349–11364.

Wang, S. Y., Yang, C. W., Liao, J. W., Zhen, W. W., Chu, F. H., & Chang, S. T. (2008). Essential Oil from Leaves of *Cinnamomum osmophloeum* Acts as a Xanthine Oxidase Inhibitor and Reduces the Serum Uric Acid Levels in Oxonate-Induced Mice. *Phytomedicine*, *15*(11): 940–945.

Wang, X., Wang, C., Hu, Q., Lv, Y., Zhang, X., Ouyang, Z., & Kong, L. (2010). The dual actions of Sanmiao wan as a hypouricemic agent: Down-regulation of hepatic XOD and renal mURAT1 in hyperuricemic mice. *Journal of Ethnopharmacology*, *128*(1): 107–115.

Wang, Y., Zhang, G., Pan, J., & Gong, D. (2015). Novel insights into the inhibitory mechanism of kaempferol on xanthine oxidase. *Journal of Agricultural and Food Chemistry*, *63*(2): 526–534.

Wang, Y., Zhao, Y., Yang, F., Yuan, Y., Wang, H., & Xiao, J. (2012). Influences of Glucose on the Dietary Hydroxyflavonoid–Plasma Protein Interaction. *Journal of Agricultural and Food Chemistry*, *60*(49): 12116–12121.

Wang, Y., Zhu, J. X., Kong, L. D., Yang, C., Cheng, C. H., & Zhang, X. (2004). Administration of Procyanidins from Grape Seeds Reduces Serum Uric Acid Levels and Decreases Hepatic Xanthine Dehydrogenase/Oxidase Activities in Oxonate-Treated Mice. *Basic & Clinical Pharmacology & Toxicology*, *94*(5): 232–237.

Weaver, A. L. (2008). Epidemiology of gout. *Cleveland Clinic Journal of Medicine*, *75*(Suppl_5).

Weselman, K. O, and C. A., Agudelo. (2001) Gout basics. *Bulletin on the Rheumatic diseases. 50*(9): 1–3.

Wiart C. (2002). *Medicinal Plants of Southeast Asia, Kuala Lumpur:* Pearson Malaya Sdn Bhd.

Wilcox W. D. (1996). Abnormal serum uric acid levels in children. *The Journal of Paediatr*ics, *128*(6): 731–741.

Woodward O. M., Kottgen, A., Coresh, J., Boerwinkle, E., Guggino, W. B., & Köttgen, M. (2009). Identification of a urate transporter, ABCG2, with a common functional polymorphism causing gout. *Proceedings of the National Academy of Sciences*, *106* (25): 10338–10342.

Wu, E. Q., Patel, P. A., Yu, A. P., Mody, R. R., Cahill, K. E., Tang, J., Krishnan, E. (2008). Disease-Related and All-Cause Health Care Costs of Elderly Patients with Gout. *Journal of Manage Care Pharmacy, 14*(2): 164–175.

Wu, N., Zu, Y., Fu, Y., Kong, Y., Zhao, J., Li, X., Li, J., Wink, M., Efferth, T. (2010). Antioxidant Activities and Xanthine Oxidase Inhibitory Effects of Extracts and Main Polyphenolic Compounds Obtained from *Geranium sibiricum L. Journal of Agricultural & Food Chemistry 58*(8): 4737–4743.

Wu, X., Muzny, D. M., Lee, C. C., & Caskey, C. T. (1992). Two Independent Mutational Events in the Loss of Urate Oxidase during Hominoid Evolution. *Journal of Molecular Evolution*, *34*(1): 78–84.

Xu, Y. J., Sun, L. M., Qiu, D. F., Liu, X. J., Jiang, Z. B., & Yuan, C. S. (2008). Quinic Acid Esters and Sesquiterpenes from *Sonchus arvensis*. *Food Chemistry*, *111*(1): 92–97.

Yamaguchi, Y., Matsumura, T., Ichida, K., Okamoto, K., & Nishino, T. (2007). Human Xanthine Oxidase Changes its Substrate Specificity to Aldehyde Oxidase Type upon Mutation of Amino Acid Residues in the Active Site: Roles of Active Site Residues in Binding and Activation of Purine Substrate. *Journal of Biochemistry*, *141*(4): 513–524.

Yang, J., Guo, J., & Yuan, J. (2008). *In vitro* Antioxidant Properties of Rutin. *LWT-Food Science and Technology*, *41*(6): 1060–1066.

Yao, L., Jiang, Y., Singanusong, R., Datta, N., & Raymont, K. (2004). Phenolic acids and abscisic acid in Australian Eucalyptus honeys and their potential for floral authentication. *Food Chemistry, 86*(2): 169–177.

Ye, G., Li, Z. X., Xia, G. X., Peng, H., Sun, Z. L., Huang, C. G. (2009). A New Iridoid Alkaloid from The Flowers of *Plumeria rubra* L. cv. *Acutifolia. Helvetica Chimica Acta*, *92* (12): 2790–2794.

Yoshisue, K., Masuda, H., Matsushima, E., Ikeda, K., Nagayama, S., & Kawaguchi, Y. (2000). Tissue distribution and biotransformation of potassium oxonate after oral administration of a novel antitumor agent (drug combination of tegafur, 5–chloro– 2, 4–dihydroxypyridine, and potassium oxonate) to rats. *Drug Metabolism and Disposition*, *28*(10): 1162–1167.

Yu, Z., Fong, W. P., Cheng, C. H. (2006). The dual actions of morin (3, 5,7,2',4'– pentahydroxyflavone) as a hypouricemicagent: uricosuric effect and xanthine oxidase inhibitory activity. *Journal of Pharmacology and Experimental Therapy, 316*(1): 169–175.

Yuan, X., Gao, M., Xiao, H., Tan, C., & Du, Y. (2012). Free radical scavenging activities and bioactive substances of Jerusalem artichoke (*Helianthus tuberosus* L.) leaves. *Food Chemistry*, *133*(1): 10–14.

Zaheer, Z., A. G., Konale, K. A., Patel, S., Khan, and R. Z., Ahmed. (2010). Comparative phytochemical screening of flowers of *Plumeria alba* and *Plumeria rubra*. *Asian Journal of Pharmacology Clinical Research*, *3*(4): 88–89.

Zaheer, Z., Konale, A. G., Patel, K. A., Subur Khan, W., Farooqui, M. N. (2010). P*lumeria rubra* Linn: an Indian medicinal plant. *International Journal of Pharmacy & Therapeutics 1* (2): 116–119.

Zhao, F., Wang, L., & Liu, K. (2009). *In vitro* Anti-Inflammatory Effects of Arctigenin, a Lignan from *Arctium lappa L.*, Through Inhibition on iNOS Pathway. *Journal of Ethnopharmacology*, *122*(3): 457–462.

Zhao, X., Zhu, J. X., Mo, S. F., Pan, Y., & Kong, L. D. (2006). Effects of Cassia Oil on Serum and Hepatic Uric Acid Levels in Oxonate-Induced Mice and Xanthine Dehydrogenase and Xanthine Oxidase Activities in Mouse Liver. *Journal of Ethnopharmacology*, *103*(3): 357–365.

Zhao, Y., Zhao, J., Li, X. P., Zhou, C. X., Sun, H. D., Hao, X. J., & Xiao, P. G. (2006). [Advances in caffeoylquinic acid research]. *Zhongguo Zhong yao za zhi= Zhongguo zhongyao zazhi= China journal of Chinese materia medica*, *31*(11): 869–874.

Zhishen, J., Mengcheng, T., & Jianming, W. (1999). The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chemistry*, *64*(4): 555–559.

Zhu, J. X., Wang, Y., Kong, L. D., Yang, C., & Zhang, X. (2004). Effects of *Biota orientalis* extract and its flavonoid constituents, quercetin and rutin on serum uric acid levels in oxonate-induced mice and xanthine dehydrogenase and xanthine oxidase activities in mouse liver. *Journal of Ethnopharmacology*, *93*(1): 133–140.

Zhu, Y., Pandya, B. J., & Choi, H. K. (2011). Prevalence of gout and hyperuricemia in the US general population: The National Health and Nutrition Examination Survey 2007–2008. *Arthritis & Rheumatism*, *63*(10): 3136-3141.