CHAPTER 4

DISCUSSION

The present study has revealed antioxidative and antihypertensive activities of the Aq. *G*. The Aq. *G* exhibited an antioxidative activity when subjected to various assays including $ABTS^{\bullet+}$, superoxide anion, hydroxyl radical scavenging assay, anti-lipid peroxidation activity, metal chelating activity and reducing power.

In the $ABTS^{\bullet+}$ scavenging activity experiments, the EC_{50} value of the Aq. *G* was found to be 282.08 ± 9.74 mg/mL. The $ABTS^{\bullet+}$ radical scavenging assay has been widely used to evaluate the antioxidant activity of compounds due to the simple, rapid, sensitive, and reproducible procedure (98). $ABTS^{\bullet+}$ radical is not found in mammalian biology and thus represents a "nonphysiological" radical source. It is a blue-green chromophore produced by the oxidation of ABTS with potassium persulfate. The antioxidant activity is determined by the decolorization of the $ABTS^{\bullet+}$ through measuring the reduction of the radical cation at 734 nm. The extent of inhibition of $ABTS^{\bullet+}$ was plotted as a function of concentration in order to compared with a standard amount of Trolox and the TEAC value. It was observed that higher the TEAC value of the sample, the stronger was the antioxidant activity. The assay is also an excellent method for determining the antioxidant activity of a broad diversity of substances, such as hydrogen donating antioxidants (scavengers of aqueous phase radicals) and of chain-breaking antioxidants (scavengers of lipid peroxyl radicals) (98-100).

Superoxide anion radical is an initial free radical formed from mitochondrial electron transport systems. Mitochondria generate energy using a 4-electron chain reaction, reducing oxygen to water. Some of the electrons escaping from the mitochondrial chain reaction directly react with oxygen and form superoxide anion. Although a relatively weak oxidant, $O_2^{\bullet-}$ exhibits limited chemical reactivity, but can generate more dangerous species (other reactive oxygen species) in living systems, including hydrogen peroxide, hydroxyl radical or singlet oxygen (101), which induce oxidative damage in lipids, proteins, and DNA (102). Also, $O_2^{\bullet-}$ is an oxygen-centered radical with selective reactivity. This species are produced by a number of enzyme systems in auto-oxidation reactions and by non-enzymatic electron transfers that univalently reduce molecular oxygen. It can also reduce certain iron complexes such as cytochrome *c*. In most organisms, $O_2^{\bullet-}$ is converted to hydrogen peroxide by superoxide dismutase.

In the assay for superoxide anion radical scavenging activity, $O_2^{\bullet-}$ is induced in the PMS/NADH-NBT system. $O_2^{\bullet-}$ is derived from dissolved oxygen by PMS/NADH coupling reaction reduces NBT. In this method, $O_2^{\bullet-}$ reduces the yellow dye (NBT^{2+}) to produce the blue formazan, which is measured spectrophotometrically at 560 nm. The decrease of absorbance with antioxidants thus indicates the consumption of $O_2^{\bullet-}$ in the reaction mixture. In the present study, the EC_{50} value of $O_2^{\bullet-}$ scavenging activity, of the Aq. *G* was found to be 3.03 ± 0.23 mg/mL. Another species of the same genus: *G. tenuistipitata* var. *tenuistipitata* showed EC_{50} value of 18.08 ± 1.97 mg/mL (103).

Among the reactive oxygen species, hydroxyl radical is an extremely reactive free radical and can be formed from superoxide anion and hydrogen peroxide through the Haber-Weiss reaction ($O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + OH^- + {}^{\bullet}OH$) (104) and in the presence of metal ions, such as Cu^{2+} or Fe^{2+} via the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH$) (105). These free radicals have extremely short half-lives but are capable of causing great damage in living organisms (101, 106). It is now widely held that the mutagenic capacity of reactive oxygen species is due to the direct interaction of hydroxyl radicals with DNA. Interaction of hydrogen peroxide and

superoxide with transition metals produced DNA-damaging hydroxyl radicals (107). In this study, hydroxyl radicals were produced by incubating ferric-nitrilotriacetic acid and H_2O_2 at pH 7.4, and reacted with 2-deoxy-2-ribose to degrade it into fragments that forms a pink chromogen upon heating with TBA at low pH. The EC_{50} value of the Aq. *G* was 3.58 ± 0.24 mg/mL, whereas that of the same genus: *G. tenuistipitata* var. *tenuistipitata* was 101.34 ± 0.55 mg/mL (103).

Excessive amounts of ROS lead to oxidative stress which can initiate biomolecular oxidations resulting in cell injury, DNA damage, lipid peroxidation and cell death (108,109). Lipid peroxidation is very important process in free radicals pathology as it is so damaging to cells. The cell membrane is primarily composed of polyunsaturated fatty acids, which are particularly susceptible to attack by oxidizing radicals. In living animal cells peroxidized membranes lose their permeability, becoming rigid, reactive and nonfunctional (110). Lipid peroxidation products can inhibit protein synthesis, block macrophage action and cause changes in chemotaxis and enzymic activity (111). The reactive oxygen species such as superoxide, hydroxyl radicals seem to induce cell degeneration via peroxidation of membrane lipids, the breaking of deoxyribonucleic acid strands, and denaturing cellular proteins (112). The anti-lipid peroxidation assay is often the first parameter to prove the involvement of free radicals in cell damage. In the present study, the liver of rat was used as a source of polyunsaturated fatty acids for determining the extent of lipid peroxidation. Malondialdehyde, a lipid peroxidation product, is an indicator of reactive oxygen species (ROS) generation in the tissue (113). In the anti-lipid peroxidation assay, ascorbate acts as a pro-oxidant agent, reducing Fe^{3+} and originating Fe^{2+}. This Fe^{2+} can initiate lipid peroxidation by the Fenton reaction (114-116) as well as accelerating peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals susceptible of inducing the oxidative stress events cascade. Therefore, in the test for anti-lipid peroxidation activity, malondialdehyde are formed by oxidation of polyunsaturated fatty acids that can be reacted with two molecules of thiobarbituric acid to from thiobarbituric acid-reactive substances (TBARs) to give a pinkish red chromogen, which is measured at 532 nm and the inhibitory effects of the Aq. *G* on Fe^{2+}/ascorbic acid induced lipid peroxidation on

liver homogenates were also calculated. The Aq. *G* exhibited an ability to inhibit lipid peroxidation with the EC_{50} value of 0.97± 0.01 mg/mL.

Transition metal ions have a great importance in the generation of oxygen free radicals in living organisms. Among the transition metals, iron is known as the most important lipid oxidation pro-oxidant due to its high reactivity. However, iron is an extremely reactive metal and will catalyze oxidative changes in lipid, proteins and other cellular components and it promotes the free radical chain reactions (117). Iron exists in two distinct oxidation states; ferrous ion (Fe^{2+}) and ferric ion (Fe^{3+}). The ferric ion (Fe^{3+}) is the relatively biologically inactive form of iron. However, it can be reduced to the active Fe^{2+}, depending on the conditions, particularly pH (118), and oxidized back through Fenton type reactions, with production of hydroxyl radicals; or Haber-Weiss reactions with superoxide anions (119,120). The production of these radicals can lead to lipid peroxidation, protein modification and DNA damage. Chelating agents may inactivate metal ions and potentially inhibit the metal-dependent processes (97). Also, the production of highly ROS such as superoxide anion radicals, hydrogen peroxide, and hydroxyl radicals is also catalyzed by free iron through Haber-Weiss reaction ($O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + OH^- + {}^{\bullet}OH$) (104). Furthermore, chelated iron, such as EDTA-Fe, is also known to be active, since it can participate in iron-catalyzed reactions (121). In the assay system, ferrozine can quantitatively form complexes with Fe^{2+}, and in the presence of chelating agents, the complex formation is inhibited and the red colour of the complex fades. Measuring of the colour reduction, therefore, it is possible to estimate of the chelating activity of the co-existing chelator (122). The metal chelating capacity was significant, since it reduced the concentration of the catalysing transition metal in lipid peroxidation. It was reported that chelating agents are effective as secondary antioxidants because they reduce the redox potential thereby stabilizing the oxidized form of the metal ion. The Aq. *G* exhibits a metal chelating activity with the EC_{50} value of 26.69 ± 1.90 mg/mL.

It was suggested that the electron donating capacity, reflecting the reducing power of bioactive compounds, is associated with antioxidant activity (123). Most non-enzymatic antioxidant activities, such as the scavenging of free radicals and the

inhibition of peroxidation, are mediated by redox reactions (124). In the reducing power assay, the presence of reductants, such as antioxidant substances in the samples, causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form by donating an electron. The Fe^{3+}/ferricyanide complex (yellow colour of the test solution) changes to Fe^{2+} complex (various shades of green and blue colour) depending on the reducing power of antioxidant samples. Therefore, amount of Fe^{2+} complex can be monitored by measuring the formation of Perl's Prussian blue at 700 nm (125). An increase in absorbance of the reaction mixture would indicate an increase in the reducing capacity due to an increase in the formation of the Fe^{2+}complex. The Aq. *G* showed a reducing power activity with the gallic acid equivalent (GAE) value of 1.48 ± 0.03. Compounds with reducing power indicate that they are electron donors, and can reduce the oxidized intermediates of the lipid peroxidation process, so that they can act as primary and secondary antioxidants (126).

The Aq. *G* showed an antioxidant activity when subjected to various assays. The results obtained from various assays ($ABTS^{+\bullet}$, superoxide anion, hydroxyl radical scavenging activity, anti-lipid peroxidation, metal chelating activity, and reducing power) expressed as EC_{50}, TEAC, GAE, EDTAE values are summarized in Table 2 and Table 3, respectively. The antioxidant activity of putative antioxidants have been attributed to various mechanisms, among which are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, and radical scavenging (127-128). The hydroxyl radical is an extremely reactive free radical and can be formed from superoxide anion. The action of a peroxidation protector may be related to its iron binding capacity. Compounds with reducing power can reduce the oxidized intermediates of the lipid peroxidation process, so that they can act as primary and secondary antioxidants (126).

Phenolic compounds are plant secondary metabolites produced either from phenylalanine via the acetate pathway or from its precursor shikimic acid via the shikimate pathway. They are characterized by the presences of a phenolic ring, i.e. a benzene ring substituted with a hydroxyl group (129). The phenolic substances are known to possess the ability to reduce oxidative damage (112), probably by trapping

free radicals directly or scavenging them through a series of coupled reactions with antioxidant enzymes (130). The direct antioxidant capacity of phenolic compounds is essentially due to the ease with which a hydrogen atom from an aromatic hydroxyl group can be donated to a free radical and the stabilization on the phenoxyl radical by delocalization of unpaired electrons around the aromatic ring (131). In addition to hydrogen donation, also single electron transfer mechanism with ionization potential as the crucial determinant for electron transfer capability is involved in the antioxidant action of polyphenols. However, hydrogen donation mechanism is considered to be more important for the radical scavenging action of most polyphenols (132). They also play important role in stabilizing lipid peroxidation. Thus the phenolic substances are associated with antioxidant activity and act as antioxidants (133). The estimation of phenolic content of the Aq. *G* was done by using Folin-Ciocalteu reagent that produced blue color by reducing yellow hetero polyphosphomolybdatetungstate anions (134). For determining total phenolic contents, calibration curves were obtained using known quantities of standard gallic acid. The Aq. *G* showed the presence of phenolic substances. It is therefore likely that the phenolic substances in the Aq. *G* play roles in its antioxidant activity. The presence of phenolic substances in genus *Gracilaria* has been reported. The amount of phenolic substances in the methanol extract of *G. changii* 1 g was equaled to 5 mg of gallic acid (135). In the presence study, the gallic acid equivalent (GAE, g sample/mg gallic acid) value of the Aq. *G* (the aqueous extract of *G. fisheri*) was 0.28 ± 0.01. Other study Selamassakul *et al*. (136) reported the amount of phenolic substances of 100g of the aqueous extract of *G. fisheri* was equivalent to 46.49 mg gallic or 2.15 when expressed in term of GAE.

Previously, the Aq. *G* was found to exhibit hypotensive activity when tested in normotensive rats under anesthesia (72). The present study has also demonstrated the hypotensive activity of the Aq. *G*. in the hypertensive (L-NAME induced) rats. The Aq. *G* showed antihypertensive activity causing a dose related decrease of blood pressure of hypertensive rats. The Aq. *G* at the doses of 5-40 mg/kg caused maximal decrease in mean arterial blood pressure of 10-80%.

Intravenous administration L-NAME (a nitric oxide synthase inhibitor) in animals, humans caused wide spread vasoconstriction and hypertension, suggesting that continuous production of nitric oxide (NO) normally maintains resistance vessels in dilate state (137). L-NAME induced hypertension involves a new mechanism for the development of hypertension (138). Long-term oral administration of L-NAME in conscious Wistar rats elevated systolic blood pressure, which reached its maximum within 4 weeks (200 ± 4 mmHg), whereafter the effect remained sustained over the rest of the 8-week treatment period (139). Chronic oral administration of L-NAME (70 mg/kg/day) in the drinking water for a period of 6 weeks elevated the mean arterial blood pressure in anaesthetized Wistar rats from 114 ± 5 mmHg to 153 ± 11 mmHg (140). Furthermore, an intravenous administration of 31 mg/kg L-NAME in anesthetized rats caused increased mean arterial blood pressure to 149 ± 9 mmHg whereas that of the control without L-NAME was 110 ± 14 mmHg (141). In the present study, an intravenous administration of L-NAME at the dose of 3 mg/kg elevated both systolic and diastolic pressure accompanied by reflex bradycardia in rat under thiopental anesthesia. Mean arterial blood pressure recorded from the rats after L-NAME administration was between 180-190 mmHg whereas that of normotensive rats was between 140-150 mmHg. The hypertensive response to L-NAME reached the peak 3 min and persisted at least 60 min.

The hypotensive effect of the Aq. *G* may be resulted from its effects on blood vessels causing vasodilation and on the heart. In isolated rat atria experiment, the Aq. *G* caused decreased force (negative inotropic effect) and rate (negative chronotropic effect) of contraction. The effects were concentration dependent.

The Aq. *G* was investigated for a vasodilating effect by determining its inhibitory effect on contractions of the isolated rat aorta experiment. The contractions of the aorta were induced by phenylephrine (α_1-adrenergic agonist, 10 μM) or high K^+ (KCl, 80 mM). The Aq. *G* showed a vasodilatating or vasorelaxation activity (ability to decrease contractions) when the contractions were induced by phenylephrine as well as by high K^+. It is generally accepted that activation of α_1-adrenergic receptors by agonists such as phenylephrine causes activation of

phospholipase C and increases the hydrolysis of phosphatidylinosital 4,5-biphosphate (PtdIns 4,5-P_2) into inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 stimulates Ca^{2+} release from intracellular stores and DAG stimulates protein kinase C (PKC). In addition, α_1-adrenergic agonists enhance Ca^{2+} entry through receptor operated Ca^{2+} channels. The contraction induced by phenylephrine (an α_1-adrenergic agonist) is due to Ca^{2+} influx through receptor-operated calcium channels causing tonic contraction, and Ca^{2+} release from intracellular stores causing phasic contraction (142). High K^+ concentration causes vascular smooth muscle contraction by depolarizing cell membranes and increasing the influx of calcium from extracellular spaces through L-type voltage-operated Ca^{2+} channels (VOCs) which subsequent release of calcium from sarcoplasmic reticulum. The vasorelaxation was observed with phenylephrine as well as with high K^+ induced contractions, thus suggesting that vasodilating activity of the Aq. *G* is mediating through inhibition of intracellular release of calcium (via the α_1-adrenergic receptors system) as well as by blocking calcium channel. Additionally, since the vasodilating is greater when tested against phenylephrine than with high K^+ induced contraction, it is likely effect of the Aq. *G* is mediated mainly via the inhibition of intracellular release of calcium.

Endothelial cells lining blood vessels produce an endothelium-derived relaxing factor (EDRF) in response to many types of stimuli such as nitric oxide (NO) prostacycline etc (143). Nitric oxide is formed in functional endothelium by the activation of nitric oxide synthase (NOS), which uses L-arginine as a substrate, and its diffusion to vascular smooth muscle cells appears to activate soluble gualylate cyclase to enhance the production of cyclic guanosine monophosphate (cGMP) by conversion of guanidine triphosphate (GTP) to cGMP. The elevated cGMP in vascular smooth muscle cells causes a relaxation of smooth muscle due to reduction of cytosolic Ca^{2+} through activation of Ca-ATPase distributed in the membrane of internal stores.

The involvement of endothelium in mediating the vasodilating activity of the Aq. *G* was examined by testing its effect in endothelium denude aorta, and also by comparing the effect on denude aorta to that of intact aorta. The Aq. *G* showed vasorelaxant effect when tested against phenylephrine-induced contraction of the

endothelium denude aorta. Additionally, the vasorelaxant effect was higher in the endothelium intact than in the denude aorta. The findings suggest that the Aq. *G* possesses a vasodilating activity which involves its effects on vascular smooth muscle and endothelium, and that the endothelium plays a major role in mediating its vasodilating activity.

In summary, the present study has demonstrated an antioxidative activity of the Aq. *G*. The presence of phenolic substances in the Aq. *G* is likely to play roles in the antioxidative activity. The Aq. *G* was found to exhibit an antihypertensive activity. The cardiodepressant and vasodilating effects of the Aq. *G* are possibly participated in its antihypertensive activity. The vasodilating effect of the Aq. *G* is involved its effects on vascular endothelium and smooth muscle. Additionally, an inhibition of intracellular release of calcium (via the α_1-adrenergic receptors system) as well as a calcium channel blocking activity are proposed to be mediated its vasodilating effect.