5.0 DISCUSSIONS

In this study, the antioxidant activities of crude petroleum benzene, chloroform, methanol and water extracts of *Allium tuberosum, Apium graveolens* (L.)*, Ipomoea batatas* (L.)*, Murraya koenigii* (L.)*, Psophocarpus tetragonolobus,* and *Sauropus androgynus* were evaluated using five assays namely DPPH radical scavenging assay, reducing power assay, metal chelating assay, haemolysate catalytic assay and lipid hydroperoxide assay.

Amount of antioxidant compounds present in a test sample or antioxidant activity exhibited is affected by several factors as below:

(a) Types of solvent

There is a general principle which is “like dissolves like”. This means non polar solvent will extract non-polar substances while polar materials will be extracted by polar solvents (Peter & Amala, 1998). As extraction solvents have differing polarities and hence affinities for the compounds of plant materials other than essential oils, it is important to know that the yield as well as the solvents. The yield composition of extracts varies with the solvent polarity (Moyler *et al.*, 1992).

According to study done by Duffy and Power (2001), different extracts dissolved solvents give different antioxidant potentials. Four solvents were used in the extraction process of *Allium tuberosum, Apium graveolens* (L.)*, Ipomoea batatas* (L.)*, Murraya koenigii* (L.)*, Psophocarpus tetragonolobus,* and *Sauropus androgynus*. The solvents were selected based on the different polarity levels. Petroleum benzene was selected because it is a very non polar solution, extremely volatile and evaporates very quickly and cleanly. Petroleum benzene can extract the compounds with low polarity such as volatile oils, fats and waxes. Meanwhile, chloroform is a non polar solution and can

extract the compounds with medium polarity such as alkaloids, volatile oils and aglycones. Methanol is a polar solution ands was selected because it can extract the compounds with high polarity such as amino acids, glycosides, sugar and other polyphenols (Peter & Amala, 1998). However, methanol is not normally used for extraction from aqueous solutions because they are too soluble in water.

Water is a polar molecule. The polarity of water molecule arises due to its greater electronegativity or electron loving nature, than hydrogen. Even though the pairs of electrons are shared with the hydrogen atoms, they are not shared equally as oxygen pulls the electrons more towards itself. This gives rise to a greater negative charge on the side of oxygen atom and a positive charge on the hydrogen atoms. The polarity of water molecule makes it a universal solvent. It can dissolve salts, acids, sugars, as well as alkalis and gases. Most cell components including proteins, polysaccharides and DNA dissolve in water making it the basis of life. Thus water was selected as one of the solvents.

(b) Evaporation process

All petroleum benzene, chloroform, methanol and water crude extracts of *Allium tuberosum, Apium graveolens* (L.)*, Ipomoea batatas* (L.)*, Murraya koenigii* (L.)*, Psophocarpus tetragonolobus,* and *Sauropus androgynus* were evaporated using a rotary evaporator at 40-50 °C. This temperature was used because higher heat may damage the antioxidant compounds present in selected local vegetables. Increasing temperature increases the evaporation process. However, there is a limit action for evaporation process in the presence of heat. Heat may cause loss of volatile compound from samples (Peter & Amala, 1998). Results obtained were also supported by the study of Duffy and Power (2001).

The higher the boiling point, the lower the rate of evaporation for samples. Of the four solvents, petroleum benzene evaporated fastest followed by chloroform, methanol and water. Water has a boiling point at 100 °C whereas the petroleum benzene most commonly used in laboratories has a boiling point range of roughly 30 °C to 60 °C. Meanwhile, the chloroform and methanol have boiling points of 61.3 °C and 65 °C. The faster the speed of the rotary evaporator, the more vapor is removed and the faster the evaporation rate. A speed of 200 rpm was used for the evaporation process.

(c) Drying temperature of plant samples

Allium tuberosum, Apium graveolens (L.), *Ipomoea batatas* (L.), *Murraya koenigii* (L.), *Psophocarpus tetragonolobus,* and *Sauropus androgynus* were dried in a hot oven at 45 °C for 48 hours. The leaves of the samples were stripped from the plants and dried according to the method described by Mohd Zin and co-workers (2002) in an oven at 45 °C for 48 hours. In contrast, Mishra *et al*., 2009 used the drying temperature in oven of 35-40 °C as the leaves were dried in shade for 3 to 4 days prior to drying. High temperature might affect the stability of antioxidant compounds and destroy heat-sensitive compounds in antioxidants (Birch *et al.*, 1974). Drying not only affects the water content of the product, but also alters other physical, biological and chemical properties such as aroma, and palatability of foods (Barbosa-Canovas & Vega-Mercado, 1996). In recent years, the drying behaviours of different aromatic plants and culinary herbs like parsley (Soysal *et al*., 2006), laurel (Yagcıoglu *et al*., 1999), bay leaves (Gunhan *et al*., 2005), dill (Raghavan *et al*., 1994), mint (Park *et al*., 2002; Lebert *et al*., 1992), and purslane (Kashaniinejad & Tabil, 2004) have been studied by many investigators. Most of the conventional thermal treatments such as hot-air drying, vacuum drying, sun drying and freeze drying result in low drying rates in the falling rate period which leads to undesirable thermal degradation of the finished products (Mousa

& Farid, 2002). In addition to its long time and environmentally dependent process, sun drying is not recommended from the hygienic point of view. Disadvantages like inconsistent quality standards, contamination problems, low energy efficiency and high costs which is not a desirable situation for the food industry (Soysal & Oztekin, 2001). Therefore, as compared to the above mentioned drying techniques; oven drying offers opportunities as less drying time, uniform energy and high thermal conductivity to the inner sides of the material, space utilization, sanitation, energy saving, precise process control, fast start-up and shut down conditions with high quality finished products (Decareau, 1992; Zhang *et al*., 2006).

(e) Type of assay to evaluate antioxidant activities

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, reduction capacity and radical scavenging (Diplock, 1997; Oktay *et al.*, 2003). Numerous antioxidant methods and modifications have been proposed to evaluate different antioxidant mechanisms, to explain how antioxidant functions. An approach with multiple assays in screening work is highly advisable. Among the plethora of methods that can be used for the evaluation of antioxidant activity are Trolox equivalent antioxidant capacity (TEAC), Tartrate-resistant acid phosphatase (TRAP), Low-density lipoprotein (LDL), NN-Dimethyl-1,4-phenylenediamine (DMPD), ferric reducing ability of plasma (FRAP), oxygen radical absorbance capacity (ORAC), 1,1-diphenyl-2-picrylhydrazyl (DPPH), photochemiluminescence (PCL), and β-carotene bleaching, thus ensuring a better comparison of the results and covering a wider range of possible applications (Sacchetti *et al.*, 2005). Taking this into account, the *in vitro* antioxidant activity of crude extracts of *Allium tuberosum, Apium*

graveolens (L.), *Ipomoea batatas* (L.), *Murraya koenigii* (L.), *Psophocarpus tetragonolobus,* and *Sauropus androgynus* compared to that of ascorbic acid, BHA, EDTA, haemolysate catalase and lipid hydroperoxide as positive references standard, were evaluated using 5 different assays, namely DPPH radical scavenging assay, reducing power assay, metal chelating assay, haemolysate catalytic assay and lipid hydroperoxide assay (Liu & Yao., 2007). Each of these methods differs in the principles, characteristics and applications.

5.1 DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging assay

Radical scavenging activities are very important due to the deleterious role of free radicals in foods and in biology systems. Excessive formation of free radicals accelerates the oxidation of lipids in food and decreases food quality and consumer acceptance (Min, 1998). DPPH assay has been widely used to evaluate the free radical-scavenging effectiveness of various antioxidant substances in food systems (Cotelle *et al.*, 1996). DPPH radical scavenging methods are common spectrophotometric procedures for determining the antioxidant capacities of plant components. DPPH radical is scavenged by antioxidants through the donation of a hydrogen radical, forming the reduced DPPH-H. The chromogens are the violet. The presence of the antioxidants leads to the disappearance of the radical chromogens in DPPH (Mathew & Abraham, 2006). The intensity of the yellow colour depends on the amount and nature of radical scavenger in the sample and standard compounds. The colour changes from purple to transparent, slight yellow after reduction, which can be quantified by its decrease of absorbance at wavelength 517 nm (Huang *et al.*, 2005). The absence of antioxidant activities in the control concentration where there were no samples added is due to the optical density at 517 nm is relatively high among all other concentration. This is shown by the darkest colour which is purple after DPPH is added. The

spectrophotometer detects the dark colour and gives a high absorbance reading. In its radical form, DPPH absorbs wavelength at 517 nm, but upon reduction by antioxidant or a radical species its absorption decreases. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. Ascorbic acid was selected as standard reference in this DPPH assay because it is a potent antioxidant known to scavenge a wide variety of reactive oxygen species (ROS) (Lutsenko *et al.*, 2002).

Strong DPPH radical scavenging activity was found in the petroleum benzene and chloroform crude extract of *Murraya koenigii* (L.). Results exhibited by crude petroleum benzene and chloroform extract of *Murraya koenigii* (L.) were an effective DPPH free radical inhibitors or scavengers with the IC_{50} value of 21.25 µg/ml and 22.5 µg/ml, respectively. Relatively strong DPPH radical scavenging activity was also found in the water extracts of *Apium graveolens* (L.) and *Psophocarpus tetragonolobus* with the IC_{50} values of 25 µg/ml and 27.5 µg/ml, respectively.

Crude petroleum benzene and methanol extracts of *Allium tuberosum,* crude petroleum benzene, chloroform and methanol extract of *Apium graveolens* (L.), crude petroleum benzene extract of *Ipomoea batatas* (L.), crude methanol extract of *Murraya koenigii* (L.) and crude chloroform extract of *Psophocarpus tetragonolobus* has very weak antioxidant activities because the dark purple colour of DPPH was not reduced in any concentrations, the percentage of inhibition was less than 50% at all concentration. These extracts contained remarkably lower amounts of radical scavenging compounds and thus were weaker radical scavengers.

Absorbance of DPPH at 517 nm was selected because in the radical form, the DPPH molecule absorbs wavelength at 517 nm when DPPH accepts an electron or hydrogen radical from an antioxidant compound. Its absorption capacity decreases, it

becomes a stable diamagnetic molecule. 517 nm used therefore is a well-suited assay using DPPH radicals.

5.2 Reducing power assay

The reducing power assay is used to evaluate the ability of extracts to reduce Fe^{3+} to Fe^{2+}. In this assay, crude chloroform extract and petroleum benzene extracts of *Murraya koenigii* (L.) and water extracts of *Sauropus androgynus* showed very high reducing capacities with crude chloroform exhibiting the strongest reducing capacities. Chan *et al.*, 2008 reported that the high antioxidant activities of chloroform extracts of leafy materials were probably due to the extracted tannins and photosynthetic pigments. The chloroform crude extracts of curry leaves contained tannins may serve as a significant indicator of its potential antioxidant activity (Meir *et al.*, 1995). Mishra *et al.* (2009) reported the presence of phenolics, flavonoids and condensed tannins in both chloroform and petroleum benzene extracts of *Murraya koenigii* (L.) leaves. The presence of reductants such as antioxidant substances in the crude extracts caused the reduction of the Fe^{3+}/ ferricyanide complex to the ferrous form (Fe^{2+}). The formation Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm (Chung *et al.*, 2002). The colour change from yellow to various shades of green and blue depending on the reducing power of each antioxidant samples.

All crude extracts of *Ipomoea batatas* (L.), petroleum benzene and methanol extracts of *Sauropus androgynus*, and methanol extract of *Apium graveolens* (L.) and *Psophocarpus tetragonolobus* showed relatively weaker reducing capacity. It also means that the ability to reduce ferric (Fe^{3+}) iron to ferrous (Fe^{2+}) iron in the crude extracts were slightly weaker and the colour change to various shade of green and blue were not as obvious as the crude chloroform extract.

Crude methanol of *Apium graveolens* (L.), *Ipomoea batatas* (L.), *Psophocarpus tetragonolobus* and *Sauropus androgynus*, petroleum benzene extract of *Sauropus androgynus* and *Ipomoea batatas* (L.), chloroform and water extract of *Ipomoea batatas* (L.), showed lower reducing capacity and the reducing capacities of all these extracts are not as strong as the crude chloroform extract and petroleum benzene extracts of *Murraya koenigii* (L.) and water extracts of *Sauropus androgynus.*

The result of crude petroleum benzene extract of *Sauropus androgynus* and *Ipomoea batatas* (L.), which exhibited some reducing capacity, suggests that petroleum benzene had dissolved many volatile oils from the vegetables which can support the reduction of the ferric (Fe^{3+}) iron to ferrous (Fe^{2+}) iron.

For the reducing power activity of crude extracts in the different types of solvents, crude petroleum benzene of *Murraya koenigii* (L.) showed higher reducing capacity compared to other extracts. These results suggest that crude petroleum benzene of *Murraya koenigii* (L.) may contain the compounds with low polarity such as volatile oils and fats which can reduce ferric ion to ferrous ion in this assay. This is supported by the report by Sumit *et al*. 2009 who states that crude petroleum ether extract of *Murraya koenigii* leaves isolated of 5,8-dimethyl furanocoumarin (1) and 1-al, 3[6', 6' dimethyl 5-hexene] carbazole (2) and b-sitosterol (3) which are all low polarity compounds.

According to the data obtained, crude chloroform of *Murraya koenigii* (L.) showed stronger reducing activity than that of other extracts. These results showed that crude chloroform of other extracts only contain tannins to serve as weaker reducing capacity if compared to crude chloroform of *Murraya koenigii* (L.) which contain tannins and volatile oils to serve as better and potential antioxidant activity.

Crude methanol extracts of *Apium graveolens* (L.) exhibited stronger reducing activity than other extracts. Other than that, the reducing capacity of extracts increased with increasing concentration of crude extracts. Absorbance of the reaction mixture was read spectrophotometrically at 700 nm. Increased absorbance of the reaction mixture indicates greater reducing power. Among all of the selected vegetable, *Murraya koenigii* (L.) exhibited strongest reducing capacity.

Researchers in one study found that the reducing power assay was more accurate when measuring the antioxidant activity of water soluble antioxidants (Bub *et al.*, 2000). This accounts for the high reducing capacity of water extracts of *Saurapus androgynus. Sauropus androgynus* is an underexploited shrub that is an excellent source of vitamin A, B and C. It is also known as a blood-building agent and a cell rejuvenator. Available literature suggests that lignan diglycoside, (-)-isolariciresinol 3-alpha-O-beta-apiofuranosyl-(1->2)-O-beta-glucopyranoside, and a megastigmane glucoside, sauroposide were isolated from the aerial part of *Saurapus androgynus* together with (+)-isolariciresinol 3-alpha-O-beta-glucopyranoside, (-)-isolariciresinol 3-alpha-O-betaglucopyranoside, (+)-syringaresinol di-O-beta-glucopyranoside, guanosine and corchoionoside (Kanchanapoom *et al.*, 2003). This accounts for the strong DPPH free radical scavenging activity and ferric ions reducing power in sweet potato leaves reported by Chu *et al.*, (2000).

5.3 Metal chelating assay

Metal chelating assay evaluates the ability of test extracts to chelate ferrous ion and prevent the formation of ferrozine-Fe^{2+} complex. In the presence of other chelating agents, the complex formation was disrupted. As a result, the purple colour of the complex decreased. Measurement of the rate of colour reduction therefore allowed the estimation of the chelating activity of the co-existing chelator (Yamaguchi *et al.*, 2000).

Foods are often contaminated with transition metal ions which may be introduced by processing methods. Bivalent transition metal ions play an important role as catalysts of oxidative processes, leading to the formation of hydroxyl radicals and hydroperoxide decomposition reactions via Fenton chemistry (Halliwell, 1997). These processes can be delayed by iron chelation and deactivation. Therefore the ability of the extracts to chelate iron (Fe^{2+}) ion was evaluated and expressed as % chelation capacity.

Iron is an essential mineral for normal physiology, but an excess of it may result in cellular injury. If they undergo Fenton reaction, these reduced metals may form highly reactive hydroxyl radicals and thereby contribute to oxidative stess (Hippeli & Elstner, 1999). The resulting oxy radicals cause damage to cellular lipids, nucleic acids, proteins, carbohydrates and lead to cellular impairment. Since ferrous ions are the most effective pro-oxidants in food system, the good chelating effect would be beneficial and removal of free iron from circulation could be promising approach to prevent oxidative stress-induced diseases. When iron is chelated, it may lose pro-oxidant properties. Hence, this present study tested the chelation of Fe^{2+}, by the crude petroleum benzene, chloroform, methanol and water extracts of *Allium tuberosum, Apium graveolens* (L.), *Ipomoea batatas* (L.), *Murraya koenigii* (L.), *Psophocarpus tetragonolobus,* and *Saurapus androgynus* in a competition assay with potassium ferricyanide.

In this assay, crude methanol of *Murraya koenigii* (L.)*,* crude chloroform of *Ipomoea batatas* (L.)*,* crude water extract of *Apium graveolens* (L.) and crude petroleum benzene of *Allium tuberosum* and *Apium graveolens* (L.) was shown to be a stronger chelator. They exhibited percentage of inhibition of 88.60 ± 0.20%, 88.56 ± 0.19%, 87.46 ± 0.28%, 81.09 ± 0.27% and 81.02 ± 0.14%, respectively at the concentration of 1 mg/ml. The chelating activities were seen to increase with increasing concentration of the extracts.(Table 4.22)

The chelating effect on ferrous ion was much weaker in crude water extract of *Saurapus androgynus* and crude methanol extract of *Ipomoea batatas* (L.) and they did not exhibit more than 50% of inhibition at the concentrations evaluated. Crude water extracts of *Saurapus androgynus* inhibited only 48.14 ± 0.42% whereas crude methanol extract of *Ipomoea batatas* (L.) inhibited 46.03 ± 0.30% at 1 mg/ml indicating that compounds in this extract did not effectively interfere with formation of all ferrous and ferrozine complex. These suggest that the extracts lack metal chelators.

Metal chelating capacity is significant since antioxidants in the extract reduced the concentration of the catalyzing transition metal in lipid peroxidation (Duh *et al*., 1999). It was reported that chelating agents, which form alpha bonds with a metal and therefore are effective as secondary antioxidants because they reduce the redox potential, thereby stabilizing the oxidized form of the metal ion (Gordon, 1990).

The chelating ability of petroleum benzene extracts of *Ipomoea batatas* (L.)*, Psophocarpus tetragonolobus, Murraya koenigii* (L.) and *Saurapus androgynus*,; chloroform crude extracts of *Psophocarpus tetragonolobus, Murraya koenigii* (L.)*, Allium tuberosum, Apium graveolens* (L.) and *Sauropus androgynus*,; methanol extracts of *Allium tuberosum, Apium graveolens* (L.)*, Psophocarpus tetragonolobus,* and *Sauropus androgynus,*; water extracts of *Psophocarpus tetragonolobus*, *Murraya*

koenigii (L.), *Ipomoea batatas* (L.) and *Allium tuberosum* also exhibited percentage of inhibition more than 50% at the concentration of 1 mg/ml. The petroleum benzene crude extracts of of *Ipomoea batatas* (L.), *Psophocarpus tetragonolobus* and *Murraya koenigii* (L.) showed 79.40 ± 0.14%, 77.02 ± 0.14%, 73.61 ± 0.22% at concentration of 1 mg/ml respectively, thus they were much stronger chelator than the petroleum benzene crude extract of *Sauropus androgynus*, which only showed 66.95 ± 0.14% at the concentration of 1 mg/ml respectively. However, the chloroform crude extract of *Psophocarpus tetragonolobus, Murraya koenigii* (L.), *Allium tuberosum* showed 79.78 ± 0.13%, 78.30 ± 0.23% and 78.05 ± 0.25% at the concentration of 1 mg/ml respectively, thus they were stronger chelator than the chloroform crude extract of *Apium graveolens* (L.) and *Sauropus androgynus,* which showed 73.45 ± 0.17% and 70.99 ± 0.14% at the concentration of 1 mg/ml, respectively. This accounts for the high iron-chelating ability in chloroform crude extract of sweet potato reported by Rowena *et al.* (2008)

When compared, crude methanol extract of *Murraya koenigii* (L.) showed higher and stronger percentage of metal chelating capacity than other extracts evaluated here. It means that *Murraya koenigii* (L.) extracts effectively interfered with the formation of ferrous and ferrozine complex. This is supported by Ningappa *et al*. (2008) who reported that methanol extract of curry leaves chelated ferrous ions effectively.

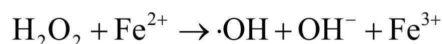
As antioxidants, flavonoids have been reported to be able to interfere with the activities of enzymes involved in reactive oxygen species generation, quenching free radicals, chelating transition metals and rendering them redox inactive in the Fenton reaction (Heim *et al*., 2002). Antioxidant compounds present in plant extracts are therefore multi-functional and their activity and mechanism would largely depend on the composition and conditions of the test system. Many authors had stressed the need

to perform more than one type of antioxidant activity measurement to take into account the various mechanisms of antioxidant action (Frankel & Meyer, 2000; Prior & Cao, 1999). Transition metal ions such as those of copper and iron are important catalysts for the generation of highly reactive hydroxyl radicals via the Fenton reaction in both in vivo and in vitro systems. Ligands that bind to metal ions can alter the redox potentials of these ions, which would render the ions catalytically silent. As secondary antioxidants, compounds can act as effective ligands that sequester copper and ferrous ions by ''wrapping'' themselves around these ions. These ligands could help intercept and suppress radicals formed via catalysis from fuelling a chain reaction (Aruoma *et al*., 1987). Free hydroxyl groups in the flavonoid ligands chelating the central metal ion may scavenge free radicals.

5.4 Haemolysate catalytic assay

Haemolysate catalytic assay evaluated the antioxidant potential of *Allium tuberosum, Apium graveolens* (L.)*, Ipomoea batatas* (L.)*, Murraya koenigii* (L.)*, Psophocarpus tetragonolobus,* and *Sauropus androgynus* based on their ability to reduce hydrogen peroxide (H_2O_2) to water and oxygen in synergism with haemolysate catalase. The absorbance at 240 nm was taken as a measure of the amount of H_2O_2 in the system as H_2O_2 possesses characteristic maximal absorbance at 240 nm. The synergistic H_2O_2-reducing activity of the plant extracts were measured based on the decrease of absorbance at 240 nm. Decreased absorbance is indicative of better reducing activity on H_2O_2 by plant extracts (Tedesco *et al*., 2001). This assay is simple, relatively inexpensive and allows rapid analysis of the synergistic effect of plant extracts with haemolysate catalase in decomposing H_2O_2.

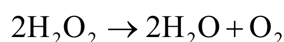
H_2O_2 is a poorly reactive and relatively harmless ROS which is naturally produced in the human body as a byproduct of aerobic metabolism. However, H_2O_2 can lead to the Fe^{2+}- catalysed generation of highly reactive hydroxyl radicals via Fenton reactions if H_2O_2 level is not well-regulated (Halliwell, 1992a).



$$H_2O_2 + Fe^{2+} \rightarrow \cdot OH + OH^- + Fe^{3+}$$

Hydroxyl radical is a potent oxidant which non-specifically reacts with cellular macromolecules, including DNA, membrane lipids and proteins. Hence, neutralization or decomposition of H_2O_2 is important in reducing the oxidative stress and preventing the pathogenesis of several diseases.

Catalase (CAT) is the primary endogenous antioxidant enzyme in the defensive mechanism of biological system which catalyses the reduction of H_2O_2 to water and oxygen (Gu *et al*., 2004).



$$2H_2O_2 \rightarrow 2H_2O + O_2$$

The role of catalase in H_2O_2 - reducing activity has been clearly demonstrated by using sodium azide in this study, in which the catalase was deprived of H_2O_2-reducing activity by treatment with catalase inhibitor sodium azide (Table 4.24, Figure 4.21). Catalase is widely distributed within the human body, with high concentrations present in the liver, kidneys and erythrocytes. Several reports have indicated that catalase is the predominant enzyme that decomposes exogenous H_2O_2 in eukaryotic cells including erythrocytes (Gaetani *et al*., 1996; Mueller *et al*., 1997).

The antioxidant enzyme catalase, by virtue of its ability to catalyse the disproportionation reaction of its substrate hydrogen peroxide, has an enormous theoretical advantage over exogenous antioxidants that are stoichiometrically consumed

(Nelson *et al*., 2006). Many botanical extracts or phytochemicals have been reported to enhance the activity of catalase and consequently reduce the oxidative stress when ingested by mammals. Therefore, the synergistic effects or the induction of endogenous antioxidant enzyme catalase by *Allium tuberosum, Apium graveolens* (L.)*, Ipomoea batatas* (L.)*, Murraya koenigii* (L.)*, Psophocarpus tetragonolobus,* and *Sauropus androgynus* extracts were investigated in this study to discover more potential sources of natural antioxidants.

5.4.1 Synergistic H_2O_2-reducing activity of *Allium tuberosum, Apium graveolens* (L.)*, Ipomoea batatas* (L.)*, Murraya koenigii* (L.)*, Psophocarpus tetragonolobus,* and *Sauropus androgynus*

In the present study, it is found that all the extracts of *Allium tuberosum, Apium graveolens* (L.)*, Ipomoea batatas* (L.)*, Murraya koenigii* (L.)*, Psophocarpus tetragonolobus,* and *Sauropus androgynus* were able to enhance the reduction of H_2O_2 in synergism with haemolysate catalase, with the crude water extract of *Psophocarpus tetragonolobus* exhibited relatively higher synergistic H_2O_2-reducing activities. The polarity of water molecule makes it a universal solvent. It can dissolve salts, acids, sugars, as well as alkalis and gases. Most cell components including proteins, polysaccharides and DNA dissolve in water. The higher synergistic H_2O_2-reducing activities in crude water extracts suggest that some of the proteins, polysaccharides and DNA have been extracted into the water and contributed to their high antioxidant potential (Peter & Amala, 1998).

The findings of this study revealed that the chloroform, methanol and water extract of *Psophocarpus tetragonolobus,* all the petroleum benzene, chloroform, methanol and water extract of *Ipomoea batatas* (L.)*,* and chloroform extract of *Murraya koenigii* (L.) have elevated better H_2O_2 - reducing activities of haemolysate catalase

compared to other extracts (Table 4.31) with percentage of reduction on H_2O_2 more than 80%. This suggests that certain components in these extracts work synergistically with haemolysate catalase to give better reducing activity on H_2O_2. It is observed that all three chloroform extract of *Psophocarpus tetragonolobus*, *Ipomoea batatas* (L.) and *Murraya koenigii* (L.) showed percentage of reduction on H_2O_2 of more than 80%. Chloroform is a solvent of intermediate polarity which adopted to extract mainly alkaloids, flavonoids, aglycones, volatile oils, triterpenes, terpenoids, tannins, and phlobatannins (Ene *et al*., 2009). This is supported by Lingli *et al*. (2007), who reported the ability of flavonoids and terpenoids to reduce H_20_2. Babalola and Areola, (2010) also reported the ability of hydrogen peroxide reduction by terpenoid extract.

The chloroform solvent may results in the higher synergistic H_2O_2-reducing activities of these extracts. The thin layer chromatography (TLC) test conducted also has showed that the chloroform extract of *Murraya koenigii* (L.) has presence of terpenoids, alkaloids compound which may have aided the synergism with haemolysate catalase to give better reducing activity on H_2O_2. This accounts for the ability of flavonoids and terpenoids to reduce hydrogen peroxide as reported by Lingli *et al*. (2007).

5.5 Lipid Hydroperoxide assay

The lipid hydroperoxide levels of *Allium tuberosum, Apium graveolens* (L.)*, Ipomoea batatas* (L.)*, Murraya koenigii* (L.)*, Psophocarpus tetragonolobus,* and *Sauropus androgynus* are measured in three types of solvent namely petroleum benzene, chloroform and methanol.

Quantification of lipid peroxidation is essential for assessing the role of oxidative injury in pathophysiological disorders. Lipid peroxidation results in the formation of highly reactive and unstable hydroperoxides of both saturated and unsaturated lipids. Peroxidation of lipids can disturb the assembly of the membrane, causing changes in fluidity and permeability, alterations of ion transport and inhibition of metabolic processes (Nigam & Schewe, 2000). In addition, Lipoprotein Lipid Hydroperoxide (LOOH) can break down, frequently in the presence of reduced metals or ascorbate, to reactive aldehyde products, including malondialdehyde (MDA), 4-hydroxy-2-nonenal (HNE), 4-hydroxy-2-hexenal (4-HHE) and acrolein (Esterbauer *et al.*, 1991; Lee *et al.*, 2001; Parola *et al.*, 1999; Uchida, K, 1999). Lipid peroxidation is one of the major outcomes of free radical-mediated injury to tissue. Peroxidation of fatty acyl groups occurs mostly in membrane phospholipids.

Traditionally, lipid peroxidation is quantified by measuring malondialdehyde (MDA) and4-hydroxynonenal (4-HNE), the degradation products of polyunsaturated fatty acid (PUFA) hydroperoxides. Sensitive colorimetric asays have been developed to measure these aldehydes. However, these assays are non- specific and often lead to an over-estimation of lipid peroxidation. There are important additional problems in using these byproducts as indicators of lipid peroxidation. The byproduct formation is highly in efficient and varies according to the transition metal ion content of the sample. Only hydroperoxides derived from PUFA give rise to these byproducts. For example, 4-HNE

is formed only from w-6 PUFA hydroperoxides and is catalysed by transition metal ions like ferrous ions. Decomposition of hydroperoxides derived from abundant cellular lipids such as cholesterol and oleic acid does not produce MDA or 4-HNE. These factors can lead to an under-estimation of lipid peroxidation. MDA is also produced in ng/ml concentrations by the platelet enzyme thromboxane synthase during whole blood clotting and platelet activation. This can lead to a gross over-estimation of lipid peroxidation.

Estimation of lipid hydroperoxides levels range from 0.3-30 μM depending on the method used. However, direct methods of estimation suggest that the concentration in normal human plasma is ~0.5 μM. Given the limitations of the indirect methods; direct measurement of hydroperoxides is the obvious choice. Hydroperoxides are the primary oxygenated products of polyunsaturated fatty acids and are key intermediates in the octadecanoid signalling pathway in plants. Lipid hydroperoxides (LHPO) were determined spectrophotometrically based on their reaction with an excess of Fe^{2+}at low pH in the presence of the dye chromogen. Triphenylphosphine mediated hydroxide formation was used to authenticate the signal generated by the hydroperoxides. The method readily detected lipid peroxidation in crude petroleum benzene, chloroform and methanol extracts of *Allium tuberosum, Apium graveolens* (L.)*, Ipomoea batatas* (L.)*, Murraya koenigii* (L.)*, Psophocarpus tetragonolobus,* and *Saurapus androgynus*.

The findings of this study revealed that all petroleum benzene, chloroform, and methanol crude extracts of *Allium tuberosum, Apium graveolens* (L.)*, Ipomoea batatas* (L.)*, Murraya koenigii* (L.)*, Psophocarpus tetragonolobus*, and *Sauropus androgynus* has some level of lipid hydroperoxide in it. Antioxidants present in the crude extracts tested can inhibit lipid oxidation by interfering with the chain reaction of peroxidation or by scavenging the reactive oxygen radicals (Halliwell & Gutteridge, 1984).

Fatty acid hydroperoxides play central roles in cell signalling and the generation of defence compounds in plants. However, to date, little information is available on the level of these compounds in plant tissues and no simple method for their detection has been reported. The primary concern during the course of this study was the desire not to generate LHPOs artefactually in tissue extracts and to ensure the stability of the putative hydroperoxides once isolated. Lipid hydroperoxides are exceptionally stable under favourable conditions, such as low temperature, dilute solution, the presence of antioxidants and the absence of catalysts such as iron salts (Gardner, 1987). The determination of LHPOs in photosynthetic tissues was viewed as potentially more problematical as light absorption by species other than lipids can initiate the formation of free radicals in sensitized photo-oxidation (Chan & Cotton, 1987). Originally it had been anticipated that photosynthetic tissues may contain higher levels of LHPO than non-photosynthetic tissues resulting from reactive oxygen species damage during the extraction process. In practice, it was found that, indeed, photosynthetic tissues do contain higher levels of LHPO than non-photosynthetic tissues. However, it is important to relate the hydroperoxide content to the total lipid content of the tissue. When the data are expressed in this way it is evident that the relative LHPO content is within the range of 0.329-3.079 nmol for all tissue types (photosynthetic and non-photosynthetic) and this value represents the basal level of oxidation of membrane polyunsaturated fatty acids in plant tissues detected by this method. As shown in Table 4.39, all 18 extracts of the selected local vegetables hydroperoxide level ranges between 0.329 nmol to 3.079 nmol. Out of the 18 crude extracts, methanol extract of sweet potato leaves showed highest hydroperoxide value of 3.079 nmol whereas methanol extract of celery showed lowest hydroperoxide value of 0.329 nmol. In comparison to the lipid hydroperoxide standard, the lipid hydroperoxide values of these extracts are at satisfactory level. It is a known factor that hydroperoxides are highly reactive and

unstable. Thus, the higher the level of hydroperoxide concentration present in a sample, the more potential it is in playing a role of oxidative injury in pathophysiological disorders. Taking this into account, the methanol extract of celery is considered the safest and reliable extract compared to other tested extract as it has the lowest level of hydroperoxide value of 0.329 nmol. Although such, it is observed that all the crude extract only show low concentration of unstable and reactive hydroperoxide which ranges between 0.329 nmol to 3.079 nmol at a high concentration of 25 nmol of extract used as compared to lipid hydroperoxide standard which has 4.839 nmol of hydroperoxide level at as low as 5 nmol concentration.

In these studies, however, extraction of the samples was necessary prior to assay. In order to apply the method to the determination of LHPO present in plant tissues total lipids were first rapidly extracted in an acidified chloroform–methanol based solvent system (Bligh & Dyer, 1959; Griffiths *et al*., 1997; Lindberg & Griffiths, 1993) and the isolated lipids assayed spectrophotometrically. The method of LHPO determination reported here is a rapid and simple technique and may be useful for the determination of LHPO in tissues where the levels of these compounds may be expected to vary in plants grown under conditions of abiotic and biotic stress or during the course of senescence in plant organs.

5.6 Screening of possible group of compounds present in active crude extracts using Thin Layer Chromatography

Visualizations using thin layer chromatography were carried out to screen bioactive compounds present in the active crude extracts. Those evaluated here were crude chloroform extract of *Murraya koenigii* (L.) which showed positive and good results in 4 of the assays conducted namely DPPH Assay, Reducing Power Assay, Metal Chelating Assay and Haemolysate Catalytic Assay; crude water extract of *Psophocarpus tetragonolobus* which showed good radical scavenging activity in the DPPH Assay and good H_2O_2 - reducing activity in the Haemolysate Catalytic Assay, crude water extracts of *Apium graveolens* (L.) which showed good radical scavenging activity in the DPPH Assay and good metal chelating activity in the Metal Chelating Assay and crude petroleum benzene of *Murraya koenigii* (L.) which showed good radical scavenging activity in the DPPH Assay and good reducing activity in the Reducing Power Assay. Short wavelength UV light, long wavelength UV light, iodine vapor, sulphuric acid visulisation and Dragendorff's test were used to observe the groups of compounds present in these active crude extracts.

In this study, the pair of solvent system used was ethyl acetate with methanol where ethyl acetate is less polar and methanol is highly polar. This enabled the crude sample extract to interact with silica gel to elude the compounds. Ethyl acetate was chosen because it has a good eluting power. However, methanol is considered more effective than ethyl acetate because methanol is highly polar (Satish, Raghavendra, Mohana, & Raveesha, 2008).

Four active crude extracts underwent thin layer chromatography (TLC) with the purpose of identifying secondary compounds that are present through different types of visualizations (Yin *et al.*, 2008). The active crude extracts were spotted onto a pre

coated silica gel plate and was developed in etyhyl acetate: methanol in the ratio of (1:1) to identify the movement of spots along the TLC plate. There were no spots observed under visible light unless observed under UV light visualization and sprayed reagent. Besides, it only showed tailing under visible light that may due to the solvent system that used to develop the TLC plate. The selected pair and the ratio of solvent system in developing chamber may not be good enough in elucidating the chemical compounds that produce only a few compounds to be visible. It is because an appropriate solvent system is a critical step in the purification of natural products in a particular extracts (Friesen & Pauli, 2005). The selection of a good solvent system that is favourable to the active crude extracts is essential in order to separate the compounds present more clearly. It is because these active crude extracts might be too sensitive due to environmental changes and may change its composition.

The compounds have shown a few spots on the TLC plate with different visualization. There was a spot was present in crude chloroform extract of *Murraya koenigii* (L.), crude water extract of *Psophocarpus tetragonolobus* and crude water extracts of *Apium graveolens* (L.) under shortwave UV light indicating the presence of organic compound in these extracts and it is proved that UV light absorbs aromatic residues making them visible (Duroux *et al*., 2007).

Another spot was observed on the TLC plate of crude chloroform extract of *Murraya koenigii* (L.), crude water extract of *Psophocarpus tetragonolobus*, crude water extracts of *Apium graveolens* (L.) and crude petroleum benzene of *Murraya koenigii* (L.) under iodine vapor visualization, indicating the presence of unsaturated carbon bonds in these extracts. Amazingly, the R_f value for all the four active crude extracts were almost the same that is 0.375, 0.377, 0.379 and 0.376 for crude chloroform extract of *Murraya koenigii* (L.), crude water extract of *Psophocarpus*

tetragonolobus crude water extracts of *Apium graveolens* (L.) and crude petroleum benzene of *Murraya koenigii* (L.) respectively. TLC R_f values will often give practical predictions even with single-phase mixtures (Yin *et al*., 2008). The Dragendorff's reagent test that formed orange colour deposition indicating the presence of alkaloids compounds in all four active crude extracts of crude chloroform extract of *Murraya koenigii* (L.), crude water extract of *Psophocarpus tetragonolobus* crude water extracts of *Apium graveolens* (L.) and crude petroleum benzene of *Murraya koenigii* (L.).

Besides that, sulphuric acid visualization showed pink colour spot on TLC plate of all four active crude extracts of crude chloroform extract of *Murraya koenigii* (L.), crude water extract of *Psophocarpus tetragonolobus* crude water extracts of *Apium graveolens* (L.) and crude petroleum benzene of *Murraya koenigii* (L.). Suplhuric acid visualization is general reagent for oxidisable components (Harder & Qian, 1999). The R_f values of the spots is a parameter to predict the type of compound. From the result obtained, it is known that there are a few oxidisable components present in the active crude extracts tested.

6.0 CONCLUSION

The objectives of the study to evaluate the antioxidant potential of the crude petroleum benzene, chloroform, methanol and water extracts of *Allium tuberosum, Apium graveolens* (L.), *Ipomoea batatas* (L.), *Murraya koenigii* (L.), *Psophocarpus tetragonolobus,* and *Sauropus androgynus* in 5 different assays namely DPPH radical scavenging assay, reducing power assay, metal chelating assay, haemolysate catalytic assay and lipid hydroperoxide assay was achieved through this research. Out of the 6 selected local vegetables, crude extracts of *Murraya koenigii* (L.) showed relatively high and satisfying antioxidant potential in all the assays carried out. In the DPPH assay, petroleum benzene and chloroform extract of *Murraya koenigii* showed good free radical scavenging activities (IC_{50} = 21.5 µg/ml and 22.5 µg/ml respectively) when compared with standard ascorbic acid (IC_{50} = 3.75 µg/ml). In the reducing power assay, chloroform extract of *Murraya koenigii* showed strong reducing powers with absorbance value of 1.833 ± 0.003 when compared with standard butylated hydroxyanisole (BHA) with absorbance value of 2.625 ± 0.004 at 1 mg/ml. In the metal chelating assay, methanol extract of *Murraya koenigii* showed the highest metal chelating capacity of 88.60 ± 0.20% at 1 mg/ml when compared to ethylenediaminetetraacetic acid (EDTA) which was 98.63 ± 0.13%. In the haemolysate catalytic activity, chloroform extract of *Murraya koenigii* showed strong H_2O_2 reducing activities of 87.36 ± 0.77% at 200 µg/ml while the standard absorbance by unblocked catalase is 99.95 (0.949) ± 0.02%. In the lipid hydroperoxide assay, petroleum benzene, chloroform and methanol extract of *Murraya koenigii* showed relatively low hydroperoxide value of 1.910, 1.877 and 2.775 nmol at 25 nmol in comparison with hydroperoxide value of the lipid hydroperoxide standard which was 4.839 nmol obtained at 5 nmol. Visualisation using thin layer chromatographyn on chloroform extract of *Murraya koenigii* showed the presence of organic compounds, unsaturated

carbon bonds, alkaloid compounds and oxidisable component which may be linked to the high anti-oxidative activities observed; hence its dietary consumption may be beneficial for health maintenance.