CHAPTER 5: DISCUSSION

5.1 Sample and Extract Preparation

5.1.1 Good Agricultural and Collections Practices (GACP)

The uses of traditional medicine especially herbal medicines have been increasing worldwide during the last two decades. However, detrimental effects experienced by the patients due to the use of herbal medicines have also increased. Therefore good agricultural and good collection practices based on guidelines on good agricultural and collections practices (GACP) should be applied (World Health Organization, 2003).

Medicinal plant materials derived from the same species can show significant differences in quality when cultivated at different sites, climate, owing to the influence of soil and other factors. It is probably due to physical appearance or to variations in their constituents, the biosynthesis of which may be affected by extrinsic environmental conditions which include the geographical and ecological variables. In this study, *Ervatamia coronaria* (roots, stems and leaves) and *Tinospora crispa* (stems) plant samples were identified and collected at the same site selection each time needed in order to minimize the significant differences of quality in their bioactivity. For *Tinospora crispa*, only stem part is used since this part is thought to have medicinal value based on the previous reseach.

Time of harvesting and collection of plant materials is also an important factor which influences the production of medicinal plant materials and herbal products.

Medicinal plants were harvested and collected during the optimal time period or season. It is well known that the concentrations of biologically active chemical constituents differ with the stage of plant growth and development. For example, *Ervatamia coronaria* plant was harvested and collected during the time when photosynthesis is most active especially between 12 noon until 3 pm in the evening. Based on WHO guidelines, the best time for harvesting and collection is determined according to the quantity and quality of biologically active constituents of interest rather than total vegetative yield of the focused medicinal plant parts.

As a precaution, during harvesting care is taken to make sure no foreign matters, weeds or toxic plants are mixed with the harvested medicinal plant materials. If the harvesting occurred in wet conditions, the harvested material are transported immediately to an indoor drying facility to dry the materials so as to avoid any possible adverse effects to the quality of plant materials due to increased moisture levels which can promote microbial fermentation and mould. In addition, cutting devices and harvesters should be kept clean and stored in an uncontaminated and dry place.

The place or location of collection of the medicinal plant is also taken into consideration. Medicinal plants are not collected in or near areas and places where there are high levels of pesticides and possible contaminants. Examples of such areas are drainage, mine tailings, roadsides, garbage dumps and industrial facilities which produce toxic emissions as well as riverbanks downstream from pastures which are contaminated with microbes from animal wastes. In this study, foreign matter that adhere to plant parts and decomposed medicinal plant materials were removed and discarded upon collection.

5.1.2 Cutting, Drying and Grinding Process of Medicinal Plants

After collection, the plant materials or plant specimen were cut, dried and ground into powder form. Most researchers working on the bioactivity and chemistry of secondary plant components use dried plant material because the differences in water content will affect the solubility of subsequent separation by liquid-liquid extraction and the need of relatively stable secondary metabolic plant components especially if it to be used as an antimicrobial agent (Neube *et al*., 2008). More over, many plants are used in dry form by traditional healers.

Once the plants are harvested, it is important to process them as soon as possible. The process of cutting before drying is also important to ensure drying process will be more efficient. Since this present work focused on antioxidant activity, *Ervatamia coronaria* and *Tinospora crispa* plants were cut into small pieces but not into tiny pieces because tiny pieces have large surface area. Large surface area will result in antioxidant components being easily oxidized due to increased exposure to oxygen.

There are several ways of drying medicinal plants. They include drying in the open air (shaded from sunlight), in wire-screened rooms or building , placed in thin layers on drying frames, by direct sunlight, in drying ovens etc. Dried plant materials are easier to handle and are less prone to microbial degradation. In this research, drying was carried out in the open air (shade drying) followed by drying in an oven. Drying under direct sunlight is not encouraged since the radiation of ultraviolet light (UV) from the sun can destroy

some compounds present in the plant samples. Shade drying is preferred in order to maintain or minimize loss of colour of leaves.

Drying in the oven, the temperature was maintained at 40 °C- 45 °C for three days. Drying at high temperature will destroy some of the heat-sensitive antioxidant compounds and decrease the antioxidant activities of the plant samples. For example, isoflavone is flavanoid which is easily destroyed by heat (Kudou *et al.*, 1991). When dried, plant samples were grounded into fine powder. This is because active substances cannot be extracted out without breaking the plant cell wall. Grinding is normally carried out to increase the surface area of the samples and therefore increase the contact surface with the extracting solvent later on (Starmans & Nijhuis, 1996). As a result, more secondary metabolites will be extracted out from samples during extraction process as well as extraction period can also be shortened.

5.1.3 Extraction of Medicinal Plants

Plant extraction is also a critical stage in preparation of plant extract since it also influences the outcome of the research. During the extraction procedure, solvents diffuse into the solid plant materials and solubilise compounds with similar polarity (Green, 2004). Solvent extraction technique is based on the principle that solvents with different polarities are able to extract different secondary compounds. Polar compounds or substances will dissolve in polar solvents while non-polar compounds or substances will dissolve in non-polar solvents. In this study, simple extraction was carried out consecutively by using four

solvents at different polarities namely petroleum ether (low-polarity), chloroform (medium-polarity), methanol (high-polarity) and water (high-polarity).

Based on previous research and study, ether solvent can possibly extract alkaloids, terpenoids, coumarins and fatty acids while chloroform solvent can extract flavanoids (Perrett *et al*., 1995) and terpenoids (Ayafor *et al.,* 1994). In addition, the active component that can be possibly extracted by methanol are anthocyanins, saponins, terpenoids (Taylor *et al.*, 1996), xanthoxyllines, totarol (Kubo *et al.,* 1992), tannins (Taylor *et al.,* 1996), quassinoids (Kitagawa *et al.*, 1996), lactones (Rao *et al.,* 1993), phenones (Peres *et al*., 1997), polyphenols (Vijaya *et al.*, 1995) and flavones (Sato *et al.*, 1996; Taniguchi & Kubo, 1993). On the other hand, water could be used to extract anthocyanins (Kaul *et al.*, 1985), starches, tannins (Scalbert, 1991), saponins (De Pasquale *et al.*, 1995), terpenoids, polypeptides and lectins.

In this study, the mixtures of plant material-solvent were placed in the environmental shaker at room temperature, with the rotation speed of 220 rpm for 72 hours. Shaking the plant material with solvent will increase the rate of extraction (Eloff, 1998). Following extraction, the extracts were filtered and the filtered extracts were evaporated to concentrate crude extracts using a rotary evaporator at temperature 40-45ºC. Higher temperatures were not used because heat-sensitive compounds easily denature at high temperature as well as to keep the active substances from any changes that can affect its properties. However, for water extract freeze-dryer was used. This agrees with the study by Gurib-Fakim (2006) where aqueous extracts were freeze-dried and stored at -20°C as this low temperature reduces the degradation of the bioactive natural product.

The concentrated crude extracts were then transferred into specimen tubes and wrapped with aluminium foil to minimize the destruction of light-sensitive compound. Eventually, the concentrated crude extracts were placed in desiccators and stored in a cool and dark room in order to avoid the growth of bacteria or fungal on the extract which could result in the alteration of chemical constituent of the extract as microbes may use some of the active substances as their nutrient for growth.

5.2 Thin Layer Chromatography

The common use of Thin Layer Chromatography (TLC) is for phytochemical screening of compounds prior to larger scale separation such as column chromatography, high performance liquid chromatography and so on. Thin layer chromatography is the cheapest and simplest method of detecting plant constituents because the method is reproducible, easy to run and only requires little equipment (Marston *et al*., 1997). In the present study, stationary phase utilized aluminium silica (polar) while the mobile phase utilized solvent (fairly non-polar) which function to carry the compounds up the TLC plate as it travels up from the bottom by capillary action.

The mobile phases (solvent) used in this research involved chloroform-methanol (9:1), acetone-chloroform (7:3), and dichloromethane which have been found to be the ideal solvents to separate the plant constituents. Chloroform-methanol (9:1) was used for methanol and chloroform extracts, acetone-chloroform (7:3) was used for water extract while dichloromethane was used for petroleum ether extract in both plants. The choices of mobile phase depend on the compound in the plant itself. Different compounds will have

different solubilities and adsorption to the two phases (stationary phase and mobile phase) between which they are to be separated.

In TLC, the most important influence in retardation factor (R_f) of the compounds are their polarities relative to the stationary and mobile phases. The polar compounds stick to polar stationary phase more than fairly non-polar compounds which are carried along in the mobile phase. The more polar the compound is, the more it will be retained in the stationary phase. Conversely, the less polar the compound is the more likely it will move with the mobile phase. Separation occurred when some compounds spend longer time standing still, adsorbed in the stationary phase than others do.

Spots with the same R_f value are probably the same compound if the parameter such as solvent system, amount of material spotted, temperature, adsorbent and the thickness of the adsorbent are constant. Therefore, if two compounds have the same R_f value, they are likely (but not necessarily) the same compound but if they have different R_f value, they are definitely different compounds. Sometimes, it is necessary to aid in the visualization of the compounds. This is because most organic compounds are colorless.

Visualizing of the compounds can be done under UV light (short- wave UV lamp) In addition, there are permanent or semi-permanent methods for visualization which not only allow visualization of the compounds but also to determine what functional groups are contained within the sample. This is possible because many reagents are functional group specific. Complex molecule can have more than one functional groups resulting in the appearance of different colours on the TLC plate of same extract when sprayed with

different reagents at the same retardation factor. That explained the present result of TLC of *Ervatamia coronaria* and *Tinospora crispa* plants.

Overall result of TLC plate showed that water extract contain fewer compounds as compared to petroleum ether, chloroform and methanol extracts. This maybe because water extract was the last extraction after petroleum ether, chloroform and methanol extraction. Since the extraction was done consecutively, most of the compounds present in the plant would have already been extracted out by the previous solvent. The term unknown refers to compound undetectable with the reagents used. For the reason, another type of reagent should be used.

Bear in mind, TLC cannot determine the exact identity of the compound detected unless a standard is run on the same plate with same parameter mentioned. However, the result will not be very accurate. TLC is only useful for screening the compounds as well as to know the derivatives of compound for instance alkaloid, terpenoid, phenol, flavanoid, saponin, conjugated bond and so on. For specific identification and structural elucidation of the compound, larger scale techniques are used which include liquid chromatography mass spectrophotometer, gas chromatography mass spectrophotometer and nuclear magnetic resonance or combination of them.

5.3 Total Phenolic Content (TPC)

The Total Phenolic Content of extracts was determined using Folin-Ciocalteu method. Folin-Ciocalteu reagent is a mixture of phosphotungstic ($H_3PW_{12}O_{40}$) and

phosphomolybdic ($H_3PMo_{12}O_{40}$) acids which are reduced to blue oxides of tungstene (W_8O_{23}) and molybdene (Mo_8O_{23}) during phenol oxidation. This reaction occurs under alkaline condition in the presence of sodium carbonate. The intensity of blue colour reflects the quantity of phenolic compounds, which can be measured using spectrophotometer (Conforti *et al*., 2006).

Phenolic compounds are a class of antioxidant agents which can act as free radical terminators (Shahidi & Wanasundara, 1992) and also possess scavenging ability due to their hydroxyl group (Diplock, 1997). The antioxidant activity of phenolics is mainly due to their redox properties which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers. They may also have a metal chelating potential (Rice-Evans *et al.,* 1995). The typical phenolic compounds that possess antioxidant activity have been characterized as phenolic acids and flavonoids (Kahkonen *et al.*, 1999). Phenolic acids have been reported and implicated as natural antioxidants in vegetables, fruits and other plants. For instance, vanillic acid, ferrulic acid and caffeic acid are widely distributed in the plant kingdom (Larson, 1988).

Phenolic compounds can also act as antimicrobial agent. The important subclasses in this group of compounds which have been found to have antimicrobial activity include phenols, quinones, phenolic acids, flavonoids, flavones, flavonols, coumarins and tannins. Flavonoids, flavones and flavonols have been known to be synthesized by plants in response to microbial infection, thus it is not a surprise that they have been found *in vitro* to be effective against a wide array of microorganisms (Bennet & Wallsgrove, 1994). In addition, tannins have been traditionally used for protection of inflamed surfaces of the

mouth, treatment of wound and diarrhea (Ogunleye & Ibitoye, 2003) while coumarins exhibit indirect antimicrobial effect by stimulating macrophages (Cowan, 1999).

Figure 4.1 exhibited the contents of total phenols that were measured by Folin-ciocalteu reagent in terms of gallic acid equivalent (standard curve equation: $y = 0.0066x + 0.0102$, $r^2 = 0.9954$). The highest total phenolic content was observed in methanol extract of *Ervatamia coronaria* (leaves) with 124.61 GAE/mg followed by methanol extract of *Ervatamia coronaria* (roots) with 94.61 GAE/mg, methanol extract of *Ervatamia coronaria* (stems) with 76.64 GAE/mg and chloroform extract of *Tinospora crispa* (stems) with 71.53 GAE/mg. Most of the values are significantly different at $p<0.05$ except for some of the extracts (Table 4.22). The results suggest that most of the extracts varied significantly from one extract to another in term of TPC.

The present results showed that methanol extraction of *Ervatamia coronaria* (roots, stems and leaves) yielded higher phenolic content as compared to petroleum ether, chloroform and water extraction. On the other hand, chloroform extract of *Tinospora crispa* (stems) exhibited the highest phenolic content. The findings are in agreement with Yang *et al*. (2007) which showed that methanol extract of lotus rhizome had the highest yield and total phenolic recovery. Methanol are said to be the most suitable solvent for the extraction of phenolic compounds due to its ability to inhibit the reaction of polyphenol oxidase that causes the oxidation of phenolics and its ease of evaporation as compared to water (Yao *et al*., 2004). Similarly, Pérez *et al*. (2007) reported that methanol was the most efficient solvent as compared to ethanol and water for extracting phenolic compounds from rosemary leaves. The present results also in agreement with TLC screening of methanol

extract *Ervatamia coronaria* (roots, stems and leaves) and chloroform extract of *Tinospora crispa.*

5.4 Antioxidant Activity

In this research, four different assays were used to evaluate antioxidant activities of *Ervatamia coronaria* (roots, stems and leaves) and *Tinospora crispa* (stems). Each of the assays employs different mechanism and reaction to detect antioxidant compounds. There is no single assay which will accurately reflect all antioxidants and radical sources in such an intricate system due to their complex composition (Nuutila *et al.,* 2003). It agrees with Prior *et. al.* (2005) that there is no simple universal antioxidant assay by which antioxidant capacity can be measured accurately and quantitatively. Therefore, an approach with multiple assays is highly advisable in evaluation of antioxidant capacity of biological samples to measure various oxidation products. DPPH Radical Scavenging Assay is used to analyze the capabilities of extracts to scavenge free radical of DPPH, Reducing Power Assay is used to study the capabilities of extracts to reduce Fe^{3+} ion from ferricyanide complex to ferrous ion (Fe^{2+}), Metal Chelating Assay is to evaluate the abilities of extracts to chelate ferrous ion (Fe^{2+}) and Haemolysate Catalytic Assay evaluates the ability of test samples to enhance the reduction of hydrogen peroxide (H_2O_2) into oxygen and water in synergism with haemolysate catalase.

5.4.1 DPPH Radical Scavenging Assay

2,2-Diphenyl-1-picryl hydrazyl (DPPH) is a stable nitrogen-centered free radical which has an unpaired valence electron at one atom of nitrogen bridge. Scavenging of DPPH free radical is the basis of the popular DPPH antioxidant assay. DPPH Radical Scavenging Assay is a decolorization assay that will measure the capacity of antioxidants to directly scavenge DPPH radicals by monitoring its absorbance using spectrophotometer at 517 nm wavelength. DPPH radical produce dark people colour solution in solvent such as methanol. In the presence of R-H (hydrogen/electron donating compound) or antioxidant, DPPH radical will be reduced to its non-radical form giving rise to the colour ranging from yellow to colourless, the absorption intensity also decreased according to the number of electron captured (Markowicz Bastos *et al*,. 2007)

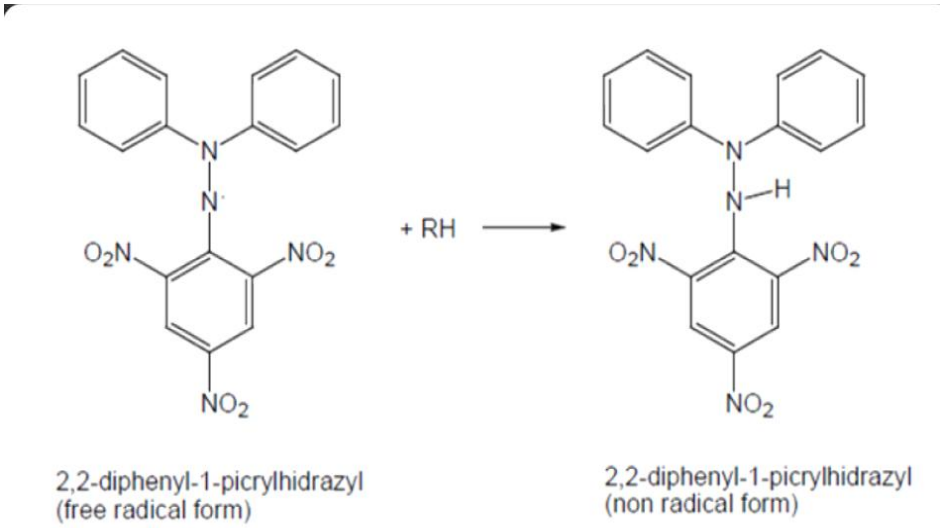


Figure 5.1 : Structure of DPPH and its reduction form by the antioxidant RH

In this spectroscopic method, the term “percentage inhibition of free radical” or “radical scavenging activity” was employed. The absorbance of the reaction mixture containing the DPPH radical and the antioxidant sample is compared to the absorbance of

the reaction mixture without any antioxidant. However, this way in expressing the result cannot be used easily due to difficulty in comparing the compounds and foods that exhibited different antioxidant properties using same concentration. Results are also expressed using the more appropriate IC_{50} parameter which can be defined as the concentration required to scavenge 50 % of the free radicals.

The lower IC_{50} value indicates the greater radical scavenging activity under the same testing conditions. Ascorbic acid was used as a positive standard in this assay due to doubts concerning the direct determination of DPPH obtained from calibration curve (Molyneux, 2004). More over, ascorbic acid has already proven as a good antioxidant and radical scavenger. Previous research by Brand-Williams *et al.* (1995), Kim *et al.* (2002), Lu and Foo (2000), and Sanchez-Moreno *et al*. (1999) also used ascorbic acid as standard for DPPH radical scavenging assay. The IC_{50} for ascorbic acid in this assay was 0.004 mg/ml with percentage of inhibition 96.97 % at 0.5 mg/ml. The concentration of standard and extracts used in this assay were 0.0025, 0.0125, 0.0375, 0.125, 0.25 and 0.5 mg/ml.

The DPPH radical scavenging activity or percentage of inhibition of free radical for *Ervatamia coronaria* (roots, stems and leaves) and *Tinospora crispa* (stems) increased with the increasing concentrations of the extracts. Chloroform extract of *Ervatamia coronaria* (roots) exhibited the highest inhibition of DPPH radical scavenging activity as compared to other extracts of the same part of plant with percentage of inhibition of 76.53% (IC_{50}=0.160 mg/ml) at 0.5 mg/ml. Conversely, methanol extract exhibited the highest inhibition of DPPH radical scavenging activity in *Ervatamia coronaria* stems and leaves with percentage of inhibition of 82.78% (IC_{50}=0.176 mg/ml) and 90.04% (IC_{50}=0.162 mg/ml)

respectively. The present result supported the study by Gupta *et al*. (2004) who also reported that methanol extract of *Ervatamia coronaria* (leaves) had significant scavenging effects on the DPPH radical and the effects increased with increasing concentration. However, all the extracts were considered as having low scavenging activity if compared to standard ascorbic acid at all concentrations tested.

The radical scavenging activity of *Tinospora crispa* (stems) also exhibited the same pattern with *Ervatamia coronaria* where the antioxidant activity increased in a dose dependent manner. Methanol extract of *Tinospora crispa* exhibited the highest inhibition of DPPH radical scavenging activity with percentage of inhibition of 90.85% (IC_{50}=0.118 mg/ml) followed by chloroform extract (59.90%, IC_{50}=0.144 mg/ml) at 0.5 mg/ml when compared with other extracts. This is in agreement with the study by Cavin *et al*. (1998) who found the presence of three compounds identified as N-cis-feruloyltyramine, N-trans-feruloyltyramine and secoisolariciresinol which exhibited antioxidant and radical scavenging properties towards 2,2,-diphenyl-1-picrylhydrazyl (DPPH) in methanol extract of *Tinospora crispa*.

Nevertheless, standard ascorbic acid is still the best radical scavenger with percentage of inhibition of 96.97% (IC_{50}=0.004 mg/ml) at 0.5 mg/ml. The IC_{50} for petroleum ether and water extracts of both plants (*Ervatamia coronaria and Tinospora crispa*) cannot be computed since the percentage of inhibition of DPPH radical scavenging activity was less than 50% at all concentrations tested.

Overall result of DPPH radical scavenging activity assay showed methanol extract was the best solvent to extract radical scavengers compound from both plants except for *Ervatamia coronaria* (roots) which showed that chloroform was the best solvent of extraction of free radical scavengers. Methanol was a suitable solvent for the DPPH assay as methanol implies better sensitivity towards the absorbance value of the DPPH (Sharma & Bhat, 2009). Besides, methanol is suitable to be used in DPPH assay with antioxidant activity of non-polar/ less polar and polar compound in the crude extracts. The study by Sharma and Bhat (2009) also mentioned that methanol solvent seems to have better IC_{50} value which may be due to the polarity of the reaction medium, chemical structure of the radical scavenger and the pH of the reaction medium.

There are some precaution steps that should be taken into consideration while doing this assay. For instance, all operation must be done in dark or dim light (Ozcelik *et, al*., 2003) to prevent sample degradation due to photo-oxidation. The recommended incubation time or reaction time should be 30 minutes and this has been followed in more recent work (Kim *et al*., 2002). However, since the rate of reaction varies widely among substrates, the best way to practice is to wait until the reaction has gone to completion or plateau (Lu *et al.*,2000; Sanchez-Moreno *et al.*, 1999; Yepez *et al.*, 2002). In addition, the initial DPPH concentration in the cuvette should be chosen to give the absorbance values less than 1.0 (which corresponds to the light intensity being reduced not more than tenfold in passing through the sample). High concentrations of DPPH will give the absorbance beyond the accuracy of spectrophotometric measurement (Ayres, 1949; Sloane & William, 1977).

5.4.2 Metal Chelating Assay

Another method which was used to evaluate antioxidant activity of *Ervatamia coronaria* and *Tinospora crispa* plants was metal chelating assay. Transition metals such as ion can stimulate lipid peroxidation in generating hydroxyl radicals through fenton reaction and accelerate lipid peroxidation into peroxyl and alkoxyl radicals which eventually drive the chain reaction of lipid peroxidation (Zhao *et al.*, 2006a). Chelating agents may inhibit radical generations by stabilizing transition metals, consequently reducing free radical damage. Besides, some phenolic compounds exhibit antioxidant activity through the chelation of metal ions (Zhao *et al.*, 2008).

Metal chelating assay works based on how effectively the extracts compete with ferrozine for ferrous ion (Fe^{2+}). Ferrozine can form complex with ferrous ion. In the presence of antioxidant (R-H), the antioxidant will chelate ferrous ion and preventing the formation of ferrozine-fe2+ complex resulting in the reduction of purple colour of the complex. Thus, measurement of colour reduction allowed the estimation of the metal ion chelating activity (Kosem *et al.*, 2007).

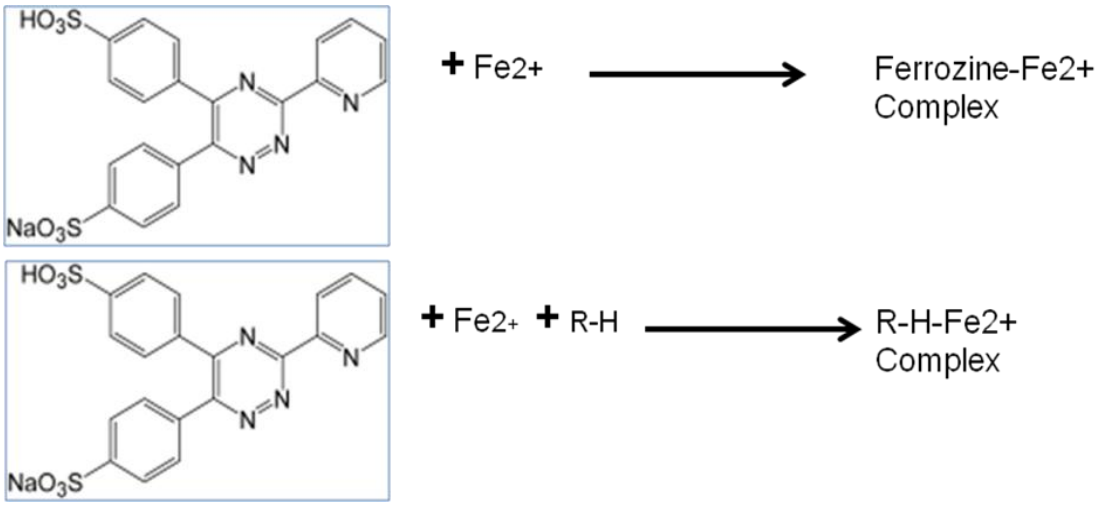


Figure 5.2 : Metal chelating reaction by antioxidant RH

In this research, EDTA (ethylenediamine tetraacetic acid) being a strong metal chelator, was used as positive reference standard (Gulcin *et al.*, 2005). EDTA structure consists of nitrogen atom and short chain carboxylic group. The percentage of inhibition of ferrozine-Fe^{2+} complex formation of EDTA increased with the increasing concentration. All the concentrations tested in this assay exhibited significantly high inhibition of ferrozine-Fe^{2+} complex. At concentration of 1 mg/ml, EDTA obtained the highest percentage of inhibition with 98.51% and the lowest percentage of inhibition was 96.27% at 0.0625 mg/ml concentration.

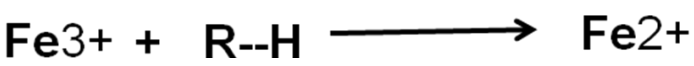
The absorbance of Fe^{2+}-ferrozine complex decreased dose-dependently where the activity was increased with increasing concentration of 0.0625 mg/ml, 0.125 mg/ml, 0.25 mg/ml, 0.5 mg/ml, and 1 mg/ml. When compared with standard EDTA, crude water extract from *Ervatamia coronaria* (roots) showed strong metal chelator with 92.62 % inhibition Fe^{2+}-ferrozine complex as well as exhibited the highest metal chelating activity in comparison with all the extracts of both plants at 1 mg/ml concentration. In addition,

chloroform extract of *Ervatamia coronaria* (stems) and methanol extract *Tinospora crispa* (stems) were also strong metal chelator with 85.24% and 81.97% inhibition of Fe^{2+}-ferrozine complex respectively while methanol extract of *Ervatamia coronaria* (leaves) exhibited moderate chelating activity with 69.64% inhibition at 1 mg/ml concentration when compared with standard EDTA. These results showed that the extract can interfere the formation of ferrous and ferrozine complex, thus suggesting the potential of the extract to chelate and capture ferrous ion before ferrozine.

All petroleum ether extracts exhibited poor metal chelator especially *Ervatamia coronaria* (leaves) with undetected percentage of inhibition due to very low metal chelating activity. The result suggested that petroleum ether solvent was not a good solvent for extracting metal chelator compound for this assay.

5.4.3 Reducing Power Assay

The third assay used to evaluate the antioxidant potential of *Ervatamia coronaria* and *Tinospora crispa* extracts was reducing power assay. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (Meir *et al.*, 1995) and is one of the methods to evaluate antioxidant activity of plants. The method works based on mechanism of reduction of Fe^{3+} from ferricyanide complex to ferrous form (Fe^{2+}) resulting in an intense blue chromogen which can be monitored by measuring the change in absorbance. In the reducing power assay, the change of yellow colour of the test solution to blue-green depends on the reducing power of each extract.



Antioxidant activity of antioxidants have been attributed to various mechanisms, among which are binding of transition metal ion catalysts, prevention of chain initiation, prevention of continued hydrogen abstraction, decomposition of peroxides, reductive capacity and radical scavenging antioxidant activity. However, reducing power assay only suitable and specific to certain antioxidant which have the ability to reduce ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) form. The change in absorbance is directly proportional related to the total reducing power of the electron donating antioxidants present in the reaction mixture (Benzie & Strain, 1999). The increased absorbance at 700 nm indicated increasing reducing power activity of the extracts.

In this research, butylated hydroxyanisole (BHA) was used as positive standard due to the greatest ability to reduce the rate at which other substances undergo oxidation. Therefore, it is a good antioxidant compound. The reducing power of BHA increased with increasing concentration. At concentration 1 mg/ml, BHA had the highest ability to reduce Fe^{3+} with 2.794 A followed by 2.610 A, 1.719 A, 1.044 A and 0.557 A at concentration 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml and 0.0625 mg/ml respectively. Higher absorbance indicated greater reducing power.

The reducing power of *Ervatamia coronaria* and *Tinospora crispa* increased with increasing concentrations. The higher reducing power reflects the higher amount of antioxidant extracted from the plant samples with the ability of reducing the oxidized intermediates of lipid peroxidation processes, therefore can act as antioxidants (Yen &

Chen, 1995). Nevertheless, the reducing power for each extract was low when compared with reference standard BHA.

The absorbance for *Ervatamia coronaria* (roots), *Ervatamia coronaria* (stems), *Ervatamia coronaria* (leaves) and *Tinospora crispa* (stems) were 0.89 A (chloroform extract), 0.85 A (chloroform extract), 0.87 A (chloroform extract) and 0.96 (methanol extract) respectively at concentration 1 mg/ml. Low reducing activity for all the extracts at all concentrations tested are probably due to less amount of antioxidant compound in the extract which have the ability of reducing ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) form.

5.4.4 Haemolysate Catalytic Assay

Haemolysate catalytic assay was carried out to assess the ability of plant crude extracts of *Ervatamia coronaria* and *Tinospora crispa* to enhance the reduction of hydrogen peroxide (H_2O_2) into oxygen and water in synergism with haemolysate catalase. This assay is also used to evaluate antioxidant potential of *Ervatamia coronaria* and *Tinospora crispa* plants. Catalase (CAT) is a heme-containing tetrameric enzyme found in nearly all living organisms that are exposed to oxygen. It is present in high amount in the human red blood cells (haemolysate catalase) in which its primary action is to catabolize and decompose H_2O_2 to form water and molecular oxygen. One of the protective roles of CAT towards haemoglobin (Hb) in the blood is by removing over half of the H_2O_2 generated in normal human red blood cell (Gaetani *et. al*, 1989).

$$2H_2O_2 \text{ ----> } 2H_2O + O_2$$

Hydrogen peroxide is naturally produced in the human body as a byproduct of aerobic metabolism. It is poorly reactive and relatively harmless Reactive Oxygen Species (ROS). However, H_2O_2 can lead to the Fe^{2+}-catalyzed generation of highly reactive hydroxyl radicals via Fenton reactions if the level of H_2O_2 is not well-regulated (Halliwell, 1992). Hydrogen peroxide is also involved in numerous types of cell and tissue injury when present in excess (Halliwell & Gutteridge, 1999). Cells will protect themselves from H_2O_2-degrading enzymes catalase which degrades H_2O_2 into water and molecular oxygen under physiological conditions but their defense are often insufficient under pathological conditions due to imbalance between H_2O_2 degradation and H_2O_2 formation (Rosser & Gores, 1995). Therefore, synergism effect of antioxidant enzyme mimics from plant extracts and haemolysate catalase were used in this assay.

The antioxidant potential was measured based on the ability of the plant crude extracts to act in synergism with haemolysate catalase to reduce the amount of H_2O_2 in the reaction medium. The synergistic effect of *Ervatamia coronaria* and *Tinospora crispa* crude extracts with haemolysate catalase was measured using UV-spectrophotometer at 240 nm since H_2O_2 possesses characteristic maximal absorbance at 240 nm wavelength. Thus, decrease in the absorbance values indicates the synergistic effect of haemolysate and crude extracts towards the H_2O_2 or better reducing activity on H_2O_2 by plant extracts (Tedesco *et al.*, 2001). The role of haemolysate catalase (CAT) in this assay has been clearly demonstrated by using sodium azide. By referring to Figure 4.15, the action of CAT was blocked with sodium azide. Thus, confirms the role of CAT in H_2O_2-reducing activity.

The findings of this study revealed that all the crude extracts of *Ervatamia coronaria* and *Tinospora crispa* were able to enhance the reduction of H_2O_2 in synergism with CAT. The percentage of reduction of H_2O_2 in synergism with haemolysate catalase in each extract increased with increasing concentrations. Nevertheless, all the extracts exhibited relatively low H_2O_2-reduction activity in synergism with CAT with less than 50% reduction. Result obtained from the present study showed that chloroform extracts of *Ervatamia coronaria* (roots, stems and leaves) exhibited the highest synergistic reducing activity of H_2O_2 at 200 µg/ml with percentage of enhanced reduction of 32.29 %, 28.48 % and 20.89 % for 6.55 mg of haem. This suggests that chloroform is the best solvent for extraction of antioxidant compounds from *Ervatamia coronaria* which have the ability to enhance the reduction of H_2O_2 in synergism with CAT.

However, a previous *in vivo* study showed methanol extract from leaves of *Ervatamia coronaria* had a significant hepatoprotective activity by decreasing lipid peroxidation and significantly increasing the level of antioxidant agents such as glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) in dose dependent manner (Gupta *et al*., 2004). Mandal and Mukherji (2001) demonstrated that *Ervatamia coronaria* has a very good scavenging system to combat the effects of air pollution. Their results exhibited that the plant in nature have high levels of activity of antioxidant agents such as SOD, CAT, GSH, phenolic peroxidase and ascorbate peroxidase.

Meanwhile, methanol extract of *Tinospora crispa* exhibited the highest synergistic reducing activity of H_2O_2 at 200 µg/ml with percentage of reduction 36.71% compared to petroleum ether, chloroform and water extracts. It might be due to secondary metabolites of

Tinospora crispa which are mostly polar compounds and therefore dissolved in polar solvent such as methanol. However, water extract exhibited very poor synergistic reducing activity of H_2O_2 at 200 µg/ml with percentage of reduction 20.02 %. Even though water extract is also a polar solvent, the secondary metabolites which contributed to H_2O_2-reducing activity in synergism with CAT may have already dissolved in methanol solvent since the extraction procedure was done consecutively.

5.5 Correlation among Total Phenolic Content and Antioxidant Activities of *Tinospora crispa* and *Ervatamia coronaria*

Based on the table 4.44, the significant linear positive correlation was confirmed between the Total Phenolic Content and DPPH Free Radical Scavenging Assay (correlation coefficient, r = 0.788, p <0.01, n=16, coefficient of determination, r^2= 0.621). In addition, significant moderate positive correlation was observed between the Total Phenolic Content and Reducing Power Assay (correlation coefficient, r = 0.556, p <0.05, n=16, coefficient of determination, r^2= 0.309). These result indicated that about 62 percents of the antioxidant capacity in DPPH Radical Scavenging Assay and only 31 percents of antioxidant capacity in Reducing Power Assay were from the phenolic compounds based on coefficient of determination (r^2). Thus, antioxidant activity of plant is not limited to phenolic compounds. The presence of secondary metabolites such as carotenoids, vitamins and volatile oils can also contribute to antioxidant activity.

Conversely, less than 0.5 are considered weak correlation. In this case, the correlation coefficient of Total Phenolic Content and Metal Chelating was only 0.358 and p>0.05 which means no significant difference while the coefficient of determination was

only 0.128. There was also no significant difference between Total Phenolic Content and Haemolysate Catalytic Assay with r= 0.388, p>0.05, r^2= 0.151. Several studies have reported the relationship between phenolic content and antioxidant activity. Velioglu *et al* ., (1998) reported a strong relationship between the total phenolic content in certain plant products while others found no such relationship (Azlim *et al.*, 2010)

The present results also suggest that not all compounds in the plants which could scavenge DPPH radical, have ability to reduce ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) and enhance the reduction of hydrogen peroxide (H_2O_2) in synergism with haemolysate catalase are good metal chelators of Fe^{2+} ion with r= 0.282, r=0.281 and r=0.302 respectively. In other words, there were no correlations between DPPH Radical Scavenging Assay, Reducing Power Assay, Haemolysate Catalytic Assay with Metal Chelating Assay. The metal ion chelating activity of the extracts might partly depend on the functional groups and content of individual functional groups in the extracts (Zhao *et al.*, 2008).

Hence, antioxidant activity of the extracts could not be relied and predicted based on TPC only. This is due to the synergism activity of phenolic compounds with one another or with other components present in an extract which may contribute to the overall observed antioxidant activity (Ordonez *et al*., 2005).

5.6 TLC Analysis and Antioxidant Assays

TLC analysis revealed the presence of secondary metabolite such as alkaloid, terpenoid, flavanoid, phenol, saponin and conjugated bond compounds in most of the

extracts. This was indicated by the positive results obtained from the reagent used such as dragendorff reagent, vanillin-sulphuric acid reagent, anisaldehyde-sulphuric acid reagent and iodine vapour.

Results of TLC showed that most of the extracts contained high amounts of flavonoids compounds except for water extracts. That explained why water extracts exhibited weak antioxidant activity in DPPH radical scavenging assay, reducing power assay and haemolysate catalytic assay. However, water extracts exhibited moderate to strong antioxidant activity in metal chelating assay for *Ervatamia coronaria* (roots, stems and leaves) and *Tinospora crispa* (stems). It probably, there are other compound which have the ability to chelate ferrous ion and prevent the formation of ferrozine-Fe^{2+} complex besides flavonoids compound.

As mentioned earlier, the common use of Thin Layer Chromatography (TLC) is for phytochemical screening of compounds prior to larger scale separation such as column chromatography, high performance liquid chromatography, liquid chromatography mass spectrometry and so on. Therefore, isolation and identification of compounds are needed followed by bioactivity assay in order to identify the compound responsible for antioxidant activity.

5.7 Antimicrobial Activity against Sinusitis-causing Microorganisms

Antimicrobial potentials of *Ervatamia coronaria* (roots, stems,leaves) and *Tinospora crispa* (stems) were analysed using disc diffusion method against 10

microorganisms causing sinusitis (comprising of 9 bacteria and 1 fungus) namely *Klebsiella pneumonia, Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Streptococcus faecalis, Proteus mirabilis, Haemophilus influenza* (ATCC49247), *Streprococcus pneumoniae* (ATCC49619), *Moraxella catarrhalis* (ATCC23296), *and Candida albican* (ATCC10281). Bacteria play a key role in the pathology of sinusitis (synonym: rhinosinusitis). Therefore, the research on the treatment of sinusitis using medicinal plant is one of the alternatives to find the solution to sinusitis.

The growth response of the selected sinusitis-causing microorganisms isolates against crude petroleum ether, chloroform, methanol and water extract of *Ervatamia coronaria* (roots, stems and leaves) and *Tinospora crispa* (stems) were determined by the disc diffusion technique followed by minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC). The disc diffusion method for antimicrobial susceptibility testing is the most practical method and is the method of choice for average laboratory. Agar disc diffusion techniques have been widely used to assay plant extracts for antimicrobial activity (Freixa *et al.,* 1996; Salie *et al.,* 1996),

In this research, Mueller Hinton agar was used for disk diffusion assay for non-fastidious bacteria such as *Staphylococcus aureus, Streptococcus faecalis, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Proteus mirabilis*. While for fastidious bacteria, the media of Mueller Hinton + 5% defibrinated blood sheep agar, Haemophilus Test Medium and Mueller Hinton + 5% defibrinated horse blood were used for antimicrobial susceptibility testing on *Streptococcus pneumoniae, Haemophilus influenza* and *Moraxella catarrhalis* respectively. This is due to fastidious organism need

additional nutrient compared to non-fastidious organism in order to grow. Sabouraud Dextrose Agar was used for antimicrobial susceptibility testing of *Candida albican* (fungus).

Mueller Hinton is usually the medium of choice although Tryptone soy agar (Lourens *et al.*,2004) or Nutrient agar (Doughari, 2006) have sometimes been used by other researchers. However, Mueller Hinton agar medium is the only susceptibility test medium that has been validated Clinical Laboratory Standard Institute (CLSI) formerly known as National Committee for Clinical Laboratory Standards (NCCLS).

Results showed that all the extracts except *Tinospora crispa* (stems) water extract can inhibit to at least one microorganism. In comparison with other extraction, water extract was not a good solvent to extract antimicrobial compound. It agrees with Parekh *et. al*. (2006) who stated that most antimicrobial active components that have been identified are not water soluble and thus organic solvent extracts have been found to be more potent. Though, traditional healers use primarily water but plant extracts from organic solvents have been found to give more consistent antimicrobial activity compared to water extracts (Parekh *et al.,* 2005). Water soluble flavonoids especially anthocyanins have no antimicrobial significance and water soluble phenolics are only important as antioxidant compounds (Yamaji *et al.,* 2005)

Results have proven that *Ervatamia coronaria* and *Tinospora cripa* have the potential on the treatment of sinusitis because they have the ability to inhibit and kill (cidal) the common sinusitis-causing microorganism such as *Streptococcus pneumoniae,*

Haemophilus influenza, Moraxella catarrhalis and *Staphylococcus aureus.* Sinusitis can be classified into acute and chronic sinusitis. In acute sinusitis, the common isolated bacteria were *Streptococcus pneumoniae, Haemophilus influenzae,* and *Moraxella catarrhalis* (Evans *et al.*, 1975; Wald *et al.*, 1991; Lew *et al.*, 1983; Brook *et al.*, 1996). *Staphylococcus aureus* often predominates and common in chronic sinusitis.

However, the usual pathogen in acute sinusitis (eg. *S. pneumoniae, H. influenzae* and *M. catarrhalis*) are rarely recovered in chronic sinusitis (Brook, 1981 & 1989; Finegold *et. al*., 2002). Gram negative enteric rods were reported in chronic sinusitis which included *Pseudomonas aeruginosa, Klebsiella pneumoniae, Proteus mirabilis* and *Escherichia coli* (Hsu *et. al.,* 1998; Nadel *et. al.,* 1998; Bahattacharyya & Kepnes, 1999; Bolger, 1994). Nevertheless, it is still a topic of controversy whether the pathogenesis of chronic infection is due to bacteria or due to an inflammatory process in which bacteria presence is secondary. However, antimicrobials are often prescribed for treatment of this infection (Brook, 2005).

With reference to antimicrobial activity against *Haemophilus influenza* (ATCC 49247), at least one of the extract of *Ervatamia coronaria* (roots, stems and leaves) and *Tinospora crispa* (stems) were shown to inhibit the bacteria. Chloroform extract of *Ervatamia coronaria* (stems) and petroleum ether extract of *Tinospora crispa* (stems) exhibited the largest inhibition zone diameter (IZD) with 12.6 mm and 11 mm diameter respectively which also considered medium antimicrobial activity according to the classification as compared to others. The results showed that inhibition increased with increasing concentrations of the extracts.

Petroleum ether and chloroform extract of *Tinospora crispa* (stems) against *Haemophilus influenza* exhibited the lowest minimal inhibitory concentration (MIC) with 6.25 mg/m while the lowest minimal bactericidal concentration (MBC) were exhibited by petroleum ether extract of *Tinospora crispa* (stems) and *Ervatamia coronaria* (leaves) with 12.5 mg/ml. This means that petroleum ether of *Tinospora crispa* (stems) only inhibit the growth of *Haemophilus influenza* at concentration 6.25 mg/ml but kill the bacteria (cidal) at 12.5 mg/ml. As compared to *Ervatamia coronaria*, *Tinospora crispa* (stems) was shown to be a better antimicrobial agent against *Haemophilus influenzae* even though both have activities against the bacteria.

Streptococcus pneumoniae is also one of the common bacteria that cause acute sinusitis. Based on the result, only 6 extracts [Chloroform and methanol extracts of *Ervatamia coronaria* (roots), *Ervatamia coronaria* (stems) and *Tinospora crispa* (stems)] had the ability to inhibit the bacteria while none of *Ervatamia coronaria* (leaves) extracts inhibited *S. pneumoniae*. Water and petroleum ether were not good solvents for extracting antimicrobial compound against *S. pneumoniae.* This is because extracts obtained from this solvents did not exhibit any inhibition zone diameter against the bacteria.

Results showed that methanol extract of *Ervatamia coronaria* (stems) was the only active extract against *Streptococcus pneumoniae,* with inhibition zone measuring 11.3 mm diameter at 50 mg/ml. Extracts producing inhibition zone diameter $\geq$ 10 mm were considered as active by Zwadyk *et al.* (1972) and Usman *et al.* (2005). The methanol extract of *Ervatamia coronaria* (stems) exhibited the lowest MIC with 6.25 mg/ml while the chloroform extract of *Ervatamia coronaria* (roots) exhibited the lowest MIC and MBC

with 6.25 mg/ml and 12.5 mg/ml respectively. This means that chloroform extract of *Ervatamia coronaria* (roots) only inhibit the growth of *Streptococcus pneumoniae* at 6.25 mg/ml but kill the bacteria (cidal) at 12.5 mg/ml

Moraxella catarrhalis is also commonly isolated from sinusitis patients in acute sinusitis. In this research, most of *Ervatamia coronaria* (roots, stems, leaves) and *Tinospora crispa* (stems) extracts inhibited *M. catarrhalis* except for water extraction of all plant parts. This is most probably due to the non-water solubility of the antimicrobial compounds which contributed to the inhibition of *Moraxella catarrhalis*. The chloroform and methanol extracts of *Ervatamia coronaria* (stems) exhibited the largest inhibition zone diameter in comparison with other extract with 15 mm and 13.3 mm diameter respectively followed by methanol extract of *Ervatamia coronaria* (leaves) with 11 mm diameter at 50 mg/ml.

The lowest MIC of *Moraxella catarrhalis* was observed in chloroform extract of *Ervatamia coronaria* (stems) with 3.12 mg/ml which was also the lowest MIC observed in this research as well as the lowest concentration used in this assay but no MBC observed in this extract. While petroleum ether extract of *Tinospora crispa* (stems) exhibited the lowest MBC with 6.25 mg/ml. This means that chloroform extract of *Ervatamia coronaria* (stems) exhibited the lowest concentration (3.12 mg/ml) that inhibited the growth of *Moraxella catarrhalis* but none of the concentration tested can kill the bacteria. Meanwhile petroleum ether extract of *Tinospora crispa* (stems) exhibited the lowest concentration (6.25 mg/ml) that kill (cidal) the bacteria in comparison to other extracts.

Staphylococcus aureus often attributes to chronic sinusitis. Results showed that 7 out of 16 type of extracts inhibited *S. aureus.* Active inhibition resulted from methanol extract of *Ervatamia coronaria* (stems) with inhibition zone of 16.7 mm diameter at 50 mg/ml followed by petroleum ether extract of *Tinospora crispa* (stems) with 16 mm and chloroform extract of *Ervatamia coronaria* (roots) with 12.7 mm. The lowest MIC was observed in petroleum ether extract of *Tinospora crispa* with 3.12 mg/ml. Most of the extracts were not bactericidal but bacteriostatic against *S. aureus* except for methanol and chloroform extracts of *Ervatamia coronaria* (roots) which MBC was 25 mg/ml respectively.

Conversely, water extract of *Ervatamia coronaria* (roots, stems and leaves) exhibited strong and medium antimicrobial activity against gram-negative enteric rod bacteria, *Klebsiella pneumoniae* with 23.3 mm (roots), 24.7 mm (stems) and 14.7 mm (leaves) diameter of inhibition zone at 50 mg/ml. Nevertheless, none of the extracts were bactericidal against the bacteria. Only chloroform extract of *Ervatamia coronaria* (roots) has ability to inhibit *Pseudomonas aeruginosa*. The controls (antibiotic) in this research were used in order to confirm the correct microorganisms were used and to ensure no contamination due to blank filter-paper disk or the solvent used (filter paper disk saturated with solvent).

There were some precaution steps taken into consideration while doing disk diffusion assay. Aseptic technique is important to protect the researcher from infection especially from the clinical specimen and also to prevent contamination of the material under process. The procedures of antimicrobial activity were done based on the

recommendation by National Committee for Clinical Laboratory Standard (NCCLS, 2000) in order to produce reliable result. Some of the bacteria were taken from clinical isolates while some were American Type Culture Collection (ATCC).

ATCC biological standards are vital and important to assure reliability of research results, reproducibility of experimentation and consistency in the scientific method. The ATCC microbial cultures have undergone rigorous authentication testing ensuring the quality and identity of the materials. Both genotypic and phenotypic testing are applied to the holdings in the Microbiology collections. Therefore, the genotypic and phenotypic of the microorganism remain the same without changes in genetic sequence due to reaction with antibiotic and mutation.

The compound in the extracts which are responsible for the antimicrobial activity against sinusitis-causing microorganisms still remain unknown since only a few researches have focused on the antimicrobial study of these plants but not specific to the sinusitis-causing microorganisms. Therefore, it is hard to compare the result with previous study. Based on previous research and present study, most compounds that have been identified in *Ervatamia coronaria* and *Tinospora crispa* were alkaloid and phenolic compound. It might be these secondary metabolites that causing the bioactivity in these plants. Nevertheless, isolation and identification of the active principles and evaluation of possible synergism among extract components for their antimicrobial activity are needed in order to confirm this. .

5.8 TLC Analysis and Antimicrobial Assays

TLC analysis revealed the presence of secondary metabolite such as alkaloid, terpenoid, flavanoid, phenol, saponin and conjugated bond compounds in most of the extracts. This was indicated by the positive results obtained from the reagent used such as dragendorff reagent, vanillin-sulphuric acid reagent, anisaldehyde-sulphuric acid reagent and iodine vapour. There is no specific secondary metabolite which commonly contributed to antimicrobial activity.

Results of TLC showed that only a few secondary metabolites were extracted from water extraction. This explained why only 1 sinusitis-causing microorganism was inhibited by water extract of *Ervatamia coronaria* (roots, stems and leaves) and no sinusitis-causing microorganism was inhibited by *Tinospora crispa* (stems) water extract. Most antimicrobial compounds are not water soluble and thus organic solvent extracts have been found to be more potent (Parekh *et al.,* 2006).

Based on TLC analysis, most of the compounds in *Ervatamia coronaria* (roots, stems and leaves) and *Tinospora crispa* (stems) were alkaloid and phenolic compounds (flavonoids). Thus, it might be these secondary metabolites that causing the antimicrobial activity in these plants. However, further isolation and identification of compounds are needed followed by bioactivity assay in order to confirm the compound responsible for antimicrobial activity.

5.9 Limitations of Antimicrobial and Antioxidant Assays

It is difficult to compare the results obtained with published results in the literature when dealing with plant extracts. This is due to multiple factors that may influence the outcome of the results such as environmental and climatic condition in which the plant grew, choice of extraction method, choice of solvents used to solubilise the secondary metabolites and choice of assays. The nature of solvent and solvent concentration as well as polarity will affect quantity and secondary metabolite composition of an extract. The beneficial medicinal effects of plant samples may not be attributed to a single compound metabolite but a combination of the metabolite compounds producing synergistic effect.

One of the antioxidant activity assay tested in this research was haemolysate catalytic assay. As mentioned earlier, haemolysate catalytic assay was carried out to assess the ability of plant crude extracts of *Ervatamia coronaria* and *Tinospora crispa* to enhance the reduction of hydrogen peroxide (H_2O_2) into oxygen and water in synergism with haemolysate catalase. This study has made use of H_2O_2 as direct indication of catalase activity which have several limitation such as this relationship is not considering other phenolic compounds that may also be absorbed at 240 nm. Besides, the best synergism activity only valid when the activity of the other enzyme such as glutathione peroxidase be evaluated and the behaviour of the interaction with the molecules is demonstrated. Therefore, these limitations should be considered in the future work.

The amount of compounds in the plants also vary between tissues (higher concentrations occur in the bark, heartwood, roots, wound tissues and branch bases), among

species from tree to tree and from season to season (Gottlieb, 1990). Based on the research in antimicrobial activity by Mitscher *et al.* (1972), the extracts are generally richest in antibacterial agents after the flowering (sexual) stage of their growth is completed and the plants which are taken from stressful environment were particularly active.

5.10 Importance of Antioxidant and Antimicrobial Activities on the Treatment of Sinusitis

The upper respiratory tract functions to protect lower respiratory structures from chemical and biological agents in inspired air. Sinusitis can be defined as an acute or chronic inflammation of the sinuses. According to Taylor (1987), the main function of the nose is to clean inhaled air so that when the air reaches the throat, the trachea, the bronchi, and the alveoli, it is clean from all or most pollutants. The nose is very efficient in doing this job by reinserting the pollutants into the exhaled air to rid them from the nose and sinuses. Nevertheless, this expiratory cycle of removing the pollutants from the nose are not complete. The pollutants still concentrate in the nasal mucus and against the nasal mucosa which increased concentration of pollutants in the nose. This accumulation of pollutants results in direct physical and chemical irritation of the nasal mucosa and causes swelling and increased mucus production. Increases in the edema of the mucosa lead to nasal obstruction and obstruction of the sinuses (Trevino, 1996).

Although many factors contribute to inflammation in sinusitis, microbial infection plays a role in the pathogenesis of sinusitis. Infection occurs when any orifice is obstructed and secretions accumulate in the body. It agrees with the report by Brabec and Bernstein (1981) that infection in the sinuses because of the swelling and obstruction of the ostium. Therefore, the chemicals that are concentrated in the nose as part of the process that is

responsible to clean inspired air can cause chronic obstruction of the sinuses, leading to chronic infectious processes and then to chronic purulent sinusitis. The common infection is by microbes such as *Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis, Staphylococcus aureus* and so on.

Inflammation of the sinuses which lead to tissue injury also can happen due to oxidative stress. Among the most potent inflammatory mediators are free radicals that can be neutralized by antioxidants. As we know, free radicals are substances that have one or more unpaired electrons in their outer shell which are formed during various biochemical reactions of aerobic metabolism in the body. The free radicals are extremely reactive and can bind to various molecules and lead to changes in DNA bases, injury to various proteins, and lipid peroxidation of membrane lipids which eventually causing tissue injury (Yilmaz *et al.*, 2004). Free radical damage is mainly caused by superoxide radical and the hydroxyl radical (Dogru *et al.,* 2001). If under normal physiological conditions tissue injury caused by free radicals is controlled by antioxidant defense system wherein free radicals are kept in balance and stability by the system. Extreme increment in the production of free radicals or decrement in the antioxidant system causes tissue injury (Cross *et. al.*, 1987). Chronic maxillary sinusitis, laryngitis, and laryngeal cancer are among such diseases leading to lower levels of antioxidants than normal in the blood (Zalewski *et. al.,* 2000)

Therefore, antioxidant and antimicrobial activity of *Ervatamia coronaria* and *Tinospora crispa* plants are both crucial for the treatment of sinusitis. Findings of this study indeed justified the potential of *Tinospora crispa* and *Ervatamia coronaria* as source of

antimicrobial and antioxidant agents by preventing inflammation to sinus which leads to sinusitis.

5.11 Liquid Chromatography Mass Spectrometry

Liquid chromatography–mass spectrometry (LC-MS, or alternatively HPLC-MS) is an analytical chemistry technique that combines the physical separation capabilities of liquid chromatography (or HPLC) with the mass analysis capabilities of mass spectrometry. It enables the analysis of very complex samples with a high degree of confidence. Only methanol extracts of all samples were chosen for LCMS/MS analysis due to activity showed by them. Initial investigation in this research of using LCMS/MS showed that all sample extracts of methanol contain high amount of alkaloid compounds in agreement with result obtained from thin layer chromatography (TLC). The mass fragmentations of the compounds were based on journal references and ACD/Labs advanced chemometrics mass fragmentations predictive software but further confirmation of the compound detected can be done using reference standards or complimentary data from various analytical instrumentations.

Since 1974, about 66 different alkaloids of *Ervatamia coronaria* have been extracted and identified (Pratchayasakul *et al.,* 2008). Results of the present study revealed the presence of about 10 types of alkaloids in *Ervatamia coronaria* crude plant extracts. For instance, the alkaloids were apparicine, lochnericine, conodurine, conodusarine, 3-oxo coronaridine, voacristine, voafidine, voafinine, voacamine and voacangine. Six alkaloids were from the roots which were apparicine, conodurine, voacamine, voacangine, voacristine and 3-oxo coronaridine while 5 alkaloids from the stems which were

apparacine, voacamine, voacristine, 3-oxo coronaridine and conodusarine. In addition, five alkaloids from the leaves which were apparicine, lochnericine, voacristine, voafidine and voafinine. Some of the alkaloids such as apparicine and voacristine were present in all parts of plant.

Terpenoids and flavanoid were also found in *Ervatamia coronaria* crude plant extracts. For instance, campesterol can be found in the roots while catechin can be found in the leaves of *Ervatamia coronaria*. According to Rhodes (1994); alkaloids, flavonoids and terpenoids are the main secondary metabolites that exhibit many physiological and pharmacological properties in living cells. However, there are still many indole alkaloids and their derivatives whose pharmacological activities are yet to be studied which may contain beneficial pharmacological properties (Pratchayasakul *et al.*, 2008).

Apparicine was reported for the first time in cell suspension culture of *Ervatamia coronaria* (Pawelka & Stoeckigt, 1983). An *in vitro* study revealed pharmacological properties of apparicine which included antimicrobial activity against Salmonella, Shigella, Escherichia, Pseudomonas, Staphylococcus, Proteus and Corynebacterium at a concentration of 1.2 percent (Rojas & Diaz, 1977). Another *in vitro* study also demonstated the ability of apparicine to inhibit the activity of Polio III virus at the concentration of 250 µg/ml (Andrade *et al*., 2005). Meanwhile, another compound namely voacamine also demonstrated a strong antimicrobial activity against Gram-positive bacteria such as *Staphylococcus aureus* and *Bacillus subtilis* (EgyptKarawya & Aboutabl, 1979) while moderate activity against Gram-negative bacteria such as *Pseudomonas aeruginosa* and *Escherichia coli* (Van Beek *et al.*, 1984). Conodurine also exhibited moderate to strong

antibacterial activity against several human pathogen (Mairura & Schmelzer , 2006) while voacangine inhibited the growth of *Mycobacterium tuberculosis* (Rastogi, 1998).

In this research, *Ervatamia coronaria* plant crude extracts exhibited antimicrobial activity against the microorganism causing sinusitis such as *Klebsiella pneumoniae, Staphylococcus aureus, Pseudomonas aeruginosa, Haemophilus influenzae, Streptococcus pneumoniae and Moraxella catarrhalis* but no antimicrobial activity against *Escherichia coli* and Proteus eventhough previous research reported antimicrobial activity against them. The true magnitude of antibacterial activity of the active alkaloid such as apparicine, voacamine and conodurine against *Escherichia coli* and Proteus may have been masked by the presence of the other alkaloids since only plant crude extracts were used and no fractionation was done. Hence, bioassay guided fractionation of the alkaloidal fraction has to be performed in order to confirm it.

To date, no research has reported regarding the antimicrobial activity of voacristine and 3-oxo coronoridine. Voacangine and Voacristine were reported to have effect on cardiovascular system. Voacangine had negative chronotropic and inotropic activities on the spontaneously beating isolated guinea pig atrium and the electrically driven isolated guinea pig left atrium (Zetler et al., 1968.) In another study with isolated guinea pig atria, voacangine antagonized the positive chronotropic and inotropic effects of noradrenaline on the heart (Zetler and Singbartl, 1970). Meanwhile, voacristine also exhibited weak stimulating effect on central nervous system and has been shown to cause a negative chronotropic effect in rats (Zetler *et al.*, 1968). In addition, 3-oxo coronaridine compound was probably not present in the fresh or living material but is formed due to oxidation,

either during the drying process or during the extraction process. Besides, there is a few or no research regarding the pharmacological activities for other alkaloids (lochnericine, conodusarine, voafinine, voafidine) found in this study.

As mentioned earlier, flavanoid and terpenoid are the main secondary metabolites that exhibit many physiological and pharmacological properties in living cells besides alkaloid. Flavanoid is one of phenolic compounds. This study revealed the presence of catechin for the first time in this plant. Polyphenolic compounds based on catechin structure are major components in green tea and have been reported to possess a wide range of pharmacological properties with numerous health-promoting effects (Rice-Evans & Packer, 2003). For instance, catechins have shown strong antioxidant effects (Sanchez-moreno *et al*., 2000) and hence it is suggested that they are responsible for reducing the risk of diseases associated with oxidative stress. In addition, catechins have other biological effects such as anticancer and antiallergy (Kondo *et al.,* 2000).

Meanwhile, the presence of terpenoid such as campesterol in this plant also contributes to some beneficial effects. Campesterol was isolated for the first time in this plant by Rastogi and colleagues (1980). Campesterol is a phytosterol with chemical structure similar to that of cholesterol. Phytosterol is known for its glucose lowering effect (Tanaka *et al*., 2006) and also known for its total cholesterol and low-density lipid cholesterol (LDL-C) lowering effects (Patch *et al.*, 2006). Apart from that, phytosterol has an ability to reduce biomarkers for oxidative stress and inflammation, as well as to reduce cancer development by a variety of mechanisms (Bradford & Awad, 2007). Therefore, we can conclude the pharmacological activities especially antioxidant activity of this plant is

probably attributed to the presence of catechins and campesterol compounds. However, bioassay guided fractionation of the fraction has to be performed in order to confirm it.

Another plant in this research was *Tinospora crispa* (stems). The results of initial investigation of this plant using LCMS/ MS revealed the presence of 6 compounds in this plant which includes 3 phenolic compounds (apigenin, brevifolin carboxylic acid and protocatechuic acid) and 3 alkaloids (jatrorhizine, magnoflorine and norboldine). Previous works have reported the presence of apigenin and jatrorhizine in this plant (Kongkathip *et al.,* 2002; Bisset & Nwaiwu, 1984). Other compounds have not yet been reported. Magnoflorine have been found in the stems of *Tinospora cordifolia* (Kumar *et al*., 2000) but to date none have reported the presence of magnoflorine in *Tinospora crispa* although they are from Menispermaceae family.

The typical phenolic compounds that possess antioxidant activity have been characterized as phenolic acids and flavonoids. For instance, flavonoids such as apigenin have been identified as major cancer-preventive components of human diets due to free radical scavenging, their anti-oxidative and anti-inflammatory activities (Galati *et al.,* 1999). Apart from that, phenolic acids such as brevifolin carboxylic acid and protocatechuic acid were believed to possess antioxidant activity. A research by N'Guessan and colleagues (2007) on *Thonningia sanguinea* flower revealed high antioxidant activity which might be due to the presence of various acidic phenolic protons. According to Ueda and colleagues (1996), the simple phenolic protocatechuic acid is one of the major benzoic acid derivatives from edible plants and fruits and shows a strong antioxidative effect which is 10-fold higher than that of α-tocopherol.

Alkaloid compounds such as jatrorhizine from coptis plant have been previously reported to have antimicrobial, antioxidant and cytotoxic activities (Wu *et al*., 1976; Wright *et al*., 2003; Rockova *et al.,* 2004). Besides, magnoflorine alkaloid also reported to has antimicrobial activities (Verpoorte, 1998). According to research by Zhao and colleagues (2006b) on the root of *Lindera augustifolia* reported the presence of norboldine. Norboldine has been found to possess free radical scavenging activity. Hence, antioxidant and antimicrobial activities are probably associated with these compounds. Therefore, the isolation and fractionation are needed and followed by *in vitro* and *in vivo* investigations which could provide further insights into the benefits of *Ervatamia coronaria* and *Tinospora crispa* for future clinical management of many human diseases.