CHAPTER 2: LITERATURE REVIEW

2.1. Importance of bringing novel antibiotics

There has been a significant increase in the emergence of disease causing microorganisms in the recent past and this has brought about an urgent need for scientists to discover new antimicrobial compounds with variable chemical structures and mechanisms of actions. There is also another important fearful matter which is the development of serious infections caused by bacteria that have become resistant to commonly used antibiotics which has become a major global healthcare problem in the twenty first century so far. Antibiotic resistance is actually nowadays problem of the hospitals and patients and it has now caused severe infections which are difficult and expensive to diagnose and treat. The Bacteria has developed resistance in all different classes of antibiotics discovered to date (Rojas *et al.* 2003); this makes this dissertation more remarkable.

This must be pointed out that, even with optimal antibiotic use, antibacterial resistances would not decline quickly and existing resistances are unlikely to vanish but we must avoid the emergence of new strains of resistant bacteria and limit the existing antibacterial resistance (Guillemot, 1999).

2.2. Medicinal plants and the *Curcuma zedoaria* place

The idea that certain plants have healing potential and they contain what we would currently call antimicrobial compounds was well accepted before humans actually discovered the existence of microbes (Ríos and Recio, 2005). Since then, mankind have used plants to treat common infectious diseases and some of these traditional medicines are still being performed as antimicrobial treatments; for example, the usage of bearberry (*Arctostaphylos uva-ursi*) and cranberry juice (*Vaccinium macrocarpon*) are used to treat urinary tract infections, or lemon balm (*Melissa officinalis*), garlic (*Allium sativum*) and tee tree (*Melaleuca alternifolia*) has a broad-spectrum of antimicrobial agents (Heinrich and Gibbons, 2004).

It has been proved that the main compounds for antibacterial activities generally contain the essential oils of these plants rather than their extracts which bring a great and vast area of usage in the treatment for infectious pathologies in the respiratory system, urinary tract, gastrointestinal and biliary systems, as well as on the skin. For example, in the case of *Melaleuca alternifolia*, the usage of the essential oil (tee tree oil) is a common therapeutic tool to treat acne and other infectious problems of the skin (Ríos and Recio, 2005).

The experimental methods used for studying the activity of both plant extracts and essential oils were published in 1988 proposing the use of diffusion methods for studying polar compounds of small or medium molecular size and determining the antimicrobial spectrum which allowed researchers to test different compounds against one microorganism and it has been widely accepted by many research groups.

Kalemba and Kunicka (2003) reviewed the classical methods commonly used for the evaluation of the antibacterial and antifungal activities of essential oils including the agar diffusion method (paper disc and well) which was used in this work field.

Microorganism growth was reported in the presence of tested essential oils and the *in vitro* antimicrobial activity of essential oils and their mechanisms of action were well monitored and concluded (Ríos and Recio, 2005).

Curcuma zedoaria has been the subject of hundreds of published papers over the past three decades, studying its antioxidant, anti-inflammatory, cancer chemo-preventive and potentially chemotherapeutic properties. The pharmacology and putative anti-cancer abilities of *Curcuma zedoaria* extracts have been the subject of several article reviews published since 1991 (Sharma *et al*. 2005).

Only a few remarkable reports have determined the antibacterial activities of *Curcuma zedoaria* before this study (Wilson *et al. 2005*; Chen *et al.* 2008; Habsah *et al.* 2000; Ficker *et al.* 2003; Chan *et al.* 2007; Loc *et al*. 2008; Chen *et al.* 2008; Niamsa and Sittiwet, 2009).

Some studies reported micropropagation improving experiments for *Curcuma zedoaria* (Keng and Stanley, 2007; Miachir *et al*. 2004; Loc *et al.* 2005) But there has not been any reports on comparing *in vitro* and *in vivo* antibacterial activity of *Curcuma zedoaria* before this study, especially in Malaysia.

2.3. Micropropagation

2.3.1. Micropropagation and medicinal plants

When working with medicinal plants, the most common method used for propagation is through tissue culture. This is to isolate new, fresh and well organised meristems like shoot tips, new rhizomes or auxiliary buds and induce them to grow into complete plants. This system of propagation is commonly referred to as micropropagation (Rout *et al.* 2000).

2.3.2. Organized growth

Creation, development or maintenance of a defined structure is called organised growth. It occurs when plant organs such as the growing points of shoots or roots (Apical meristems), leaf initials, young flower buds or small fruits, are transferred to culture and continue to grow with their preserved structure (George, 1993).

Rout *et al*. (2000) and Wilson *et al*. (2005) mentioned the term "Tubers" while Chan *et al*. (2007), Loc *et al*. (2008), Miachir *et al*. (2004) and Chen *et al*. (2008) used the term "rhizomes" for what they had excised form the Original/Mother plant of *Curcuma zedoaria* and also stated that the propagation of this plant species has been only performed through rhizomes (Loc *et al*. 2005).

In this experiment the term "Mature rhizome buds" of *Curcuma zedoaria* is used as what we call "explant" hereafter.

2.3.3. Advantages

Current plant propagation methods for the *in vitro* system are already developed and continuing methods of conventional propagation. *In vitro* techniques have the following advantages over traditional methods:

- Cultures commence with very small pieces of plants (explants), and thereafter small shoots or embryos are propagated (hence the term 'micropropagation' is used) (George, 1993).
- Propagation is basically performed in aseptic conditions, free from pathogens. The products should be all free of any sort of diseases, bacteria, fungi and other microorganisms (George, 1993).

2.3.4. Contaminants/Decontamination

Plants are definitely attacked and polluted by foreign external fungi, bacteria, animal pests and yeasts. These organisms may be present on all external surfaces and are also likely to find their way into small crevices, such as those between bud scales and under ligules or stipules or rhizomes. It is always very important to ensure that explants are only taken from vigorous plants with no disease symptoms and no history of diseases (George, 1993).

It is necessary to remove external microorgnisms from plant tissues for most types of tissue cultures in order to reject the growth ability readily on the plant culture media by bacteria and fungi. This can be carried out with chemical disinfectants, or sterilants before cultures are performed through propagation. This is because bacteria and fungi plant culture media contains organic compounds such as sugars, amino acids and vitamins and provides a great environment for microorganisms to grow (George, 1993; Rout *et al*. 2000; Wilson *et al*. 2005; Chen *et al.* 2008).

The essential and necessary levels for an effective decontamination depend on the nature of the plant material and the type of the explants used.

In general, the part of the plant from which the explants are to be dissected is first cleaned. Underground structures such as roots, tubers and rhizomes need to be washed in water with a brush to remove any soil and other particles form it. Any dead or extra and useless tissue is removed and the material is then usually washed under running tap water or soaked for several hours in water. This has been done in the same way in this dissertation and it can help to reduce the level of strong and super contaminants and will make the microorganisms to be more exposable and liable by further subsequent treatments with sterilants.

Finally the chosen part of the plant (rhizomes) was placed into a flask or any suitable container, and then chemical treatments are applied for disinfecting (George, 1993); Sterilants are explained in 2.3.5.

After exposure to sterilant solutions, explants are washed and/or soaked in several changes of sterile water to remove all traces of the sterilising agent before being prepared and transferred to culture (George, 1993; Rout *et al*. 2000; Wilson *et al.* 2005; Chen *et al.* 2008; tables 2.1 and 2.2).

2.3.5. Surface Sterilants

Surface sterilisation can be carried out with several different germicidal reagents. Ideally, the best products are those which are cheap, non-toxic to both plants and human, and provide a wide frequency of effectiveness on a wide range of plant material.

The most commonly used materials are:

• The hypochlorite ion (hypochlorous acid),

• Simple alcohols, particularly ethanol.

The best possible contact with the plant material and the material must exist and this is what the germicide should be able to carry out. It is a routine to add a few drops of wetting agent to aqueous sterilant solutions.

Many detergents are suitable. The most common one is Tween 20® (George, 1993) which is used in this study and also in other previous works (Rout *et al.* 2000; Wilson *et al.* 2005; Chen *et al*. 2008; tables 2.1 and 2.2).

2.3.6. Hypochlorite solutions

The hypochlorite ion is usually obtained from sodium hypochlorite (NaOCl) or calcium hypochlorite. The water soluble sodium hypochlorite can be obtained from laboratory suppliers in aqueous solutions type. Most laboratories use household or industrial bleach solutions as a convenient source same solutions are used in this dissertation. The sodium hypochlorite (NaOCl) concentration which is required can vary from 0.25-2 w/v according to the plant material and the period of time which it is exposed to the compound (George, 1993).

2.3.7. Alcohols

Among the alcohols, ethanol is most widely used for sterilisation, but explants material can not always be disinfected alone in ethanol only. Alcohols are not only germicidal, but also remove surface waxes from the surface of plant tissue.

The routine is that the aqueous solution is normally combined with other sterilants. Therefore ethanol permits plant tissues to be more effectively wetted and to be penetrated by another germicide (George, 1993).

Such a pre-treatment comes with different and various time and durations based on the type of tissue or organ. The proportion of contaminant-free cultures can sometimes be increased by dipping tissue in 45-80% ethanol for a short period (e.g. three-five minutes), after soaking it in hypochlorite. In the latter case it is necessary to rinse again with sterile water. It was decided to use a 70% ethanol for this step for one minute only as the rhizomes of *Curcuma zedoaria* are so brittle and sensitive (Rout *et al.* 2000; George, 1993; Rout *et al.* 2000; Wilson *et al.* 2005; Chen *et al.* 2008; tables 2.1 and 2.2).

2.3.8. Heavy metal ions

One of the most common sterilants is mercuric chloride which has been used in some previous studies too (Loc *et al. 2005*; Keng and Stanley, 2007; tables 2.1 and 2.2). This mammalian high toxic compound need a strong caution and working experience due to the environmental hazard of mercury products (Keng and Stanley, 2007; George, 1993). Heavy metal ions can be toxic if introduced into a culture medium and so special care needs be given to remove all traces after they have been used as a sterilant (George, 1993). No heavy metal ions were used in this study as there was no enough safety equipment and a suitable environment.

2.3.9. Combined treatment

For a particular plant, to control the contamination and to overcome the infections, the most effective combined treatment may only be found by experiments achieved over many experiments. A good example is provided by Hennerty *et al.* (1988) in which the Meristem shoots of apple were decontaminated perfectly when they were placed in 95% ethanol for two minute after a brief dip in 9% calcium hypochlorite before washing. If the dormant buds were treated in the same way, the cultures which were obtained from dissected shoot tips were almost all contaminated of bacteria. Increasing the duration of the calcium hypochlorite treatment to 120 minutes or combining this with an immersion of the dissected meristems in 1% calcium hypochlorite, did not solve the problem. It was only solved by the following set of treatments:

• 95% ethanol, two minutes;

• 0.05 $HgCl_2$ plus three drops Tween 20 wetter per 100 ml, ten minutes;

• Wash with Sterile distilled water, ten minuses;

• NaOCl (1 w/v available chlorine), twenty minutes;

• Four times wash in sterile distilled water (Hennerty *et al*. 1988; George, 1993).

Previous researches (Keng and Stanley, 2007; Loc *et al.* 2005; Miachir *et al. 2004*) have used different combined methods with different times and methods which has been illustrated in tables 2.1 and 2.2.

As shown in table 2.2, the total time that the explants are exposed to the sterilants are different; for instance, Miachir *et al*. (2004) have posed their explants to 2% Solution of NaOCl for a total of fifteen minutes and did not use any other sterilants but (Keng and Stanley, 2007) have exposed their explants to 2% Solution NaOCl for a total time of twenty minutes while using 100mg/l solution $HgCl_2$ for five minutes. In the other experiment from (Loc *et al*, 2005), explants were exposed to 0.2% (w/v) $HgCl_2$ for five minutes for twenty minutes.

The most effective combined treatment for controlling contamination of a particular plant may only be found by experiment (Hennerty *et al*, 1988).

A combination method was used in this study using 70% (v/v) ethanol for one minute, 20% solution NaOCl with different durations and Tween® 20 together which will be explained more in details in (chapter 3).

Sterilants ▶ / Previous works ▼	$HgCl_2$	Ethanol	NaOCl	Tween 20	Other
(Habsah *et al.* 2000) *	✘	✘	✘	✘	✘
(Miachir *et al.* 2004) Plant : *Curcuma zedoaria Roscoe*	✘	✘	👍 2 % Solution	✘	✘
(Loc *et al.* 2005) Plant : *Curcuma zedoaria Roscoe*	👍 0.2% (w/v)	👍 70% (v/v)	✘	✘	✘
(Wilson *et al. 2005*) *	✘	✘	✘	✘	✘
(Keng and Stanley, 2007) Plant : *Curcuma roscoe* and *Zingiber zerumbet*	👍 100 mg/l Solution	✘	👍 20% Solution + 10 % solution	👍 Few drops	✘
(Loc *et al*. 2008) *	✘	✘	✘	✘	✘
(Chen *et al.* 2008) *	✘	✘	✘	✘	✘
(Niamsa and Sittiwet, 2009) *	✘	✘	✘	✘	✘
This study	✘	👍 70% (v/v)	👍 20% Solution	👍 Few drops	✘

Table 2.1 Comparing and summarizing earlier reports and this study base on their use of different sterilants and concentrations.
* The experiment did not include any micropropagation and sterile treatments

Total time exposed to the Sterilants ► / Previous works ▼	$HgCl_2$ time	Ethanol time	NaOCl time	Tween 20 time
(Miachir *et al.* 2004) Plant : *Curcuma zedoaria Roscoe*	✘	✘	2 % Solution 5 minutes ↓ 3 X water ↓ 10 minutes ↓ 3 X water	✘
(Loc *et al.* 2005) Plant : *Curcuma zedoaria Roscoe*	0.2% (w/v) 20 minutes ↓ 5 X water	✪ 70% (v/v) 1 minute ↓ Subsequently ←used $HgCl_2$	✘	✘
(Keng and Stanley, 2007) Plant : *Curcuma roscoe* and *Zingiber zerumbet*	✪ 100 mg/l Solution 5 minutes ↓ 3 X water ↓ Used NaOcl ➔	✘	20% Solution 10 minutes ↓ 3 x water ↓ 20% Solution 10 minutes ↓ 3 X water	Few drops
This study	✘	70% (v/v) 1 minute ↓ 3 x water	✪ 20% Solution 5 minutes ↓ 3 x water ↓ 20% Solution 15 minutes ↓ 3 x water ↓ ←Used Ethanol	Few drops

Table 2.2 Time and preferences on use of the sterilants in earlier reports and this study
✪ Begin point for sterilizing

2.3.10. Media

Plant tissues and organs for an *in vitro* micropropagation grow on artificial media which is supplied with nutrients necessary for growth. The success level of a plant tissue culture work is strongly influenced by the environment and structure of the culture medium. For healthy and vigorous growth, intact plants need large amounts of some inorganic elements (the so-called major plant nutrients) salts of nitrogen (N), potassium (K), calcium (Ca), phosphorus (P), magnesium (Mg) and sulphur (S); and small quantities of other elements (minor plant nutrients or trace elements) like iron (Fe), Sodium (Na). Chlorine (Cl), Manganese (Ma), Zinc (Zn), boron (B), Copper (Cu), molybdenum (Mo) and (possibly) nickel (Ni) (George, 1993).

Plant tissue culture media is therefore made up from some or all of the following components:

- Macronutrients (always used)
- Micronutrients (most of time is used; sometimes just iron, has been used)
- Vitamins (can be included/not included when purchase a ready media from suppliers)
- Amino acids and other nitrogen supplements
- sugars
- A solidifying agent (Agar is the most common agent)

Previous studies have mentioned different type of media and additional supplements which are illustrated in table 3.

Ready commercial MS media (Murashige and Skoog, 1962) with vitamins was used in this study due to the lack of some ingredients for making a manual media and also the cost of making the media in the lab.

Previous studies	Media type	Additional supplements	solidifying agent
(Loc *et al*. 2005)	MS medium	Sucrose + Coconut water (CW) + AC	Agar
(Miachir *et al.* 2004)	Modified MS medium	Nicotinic acid + Thiamine and Pyridoxine + Glycine + Myo-inositol, + Sucrose +	Agar
(Keng and Stanley, 2007)	MS medium	?	?
This study	MS medium	Vitamins + Sucrose + Activated Charcoal (AC)	Phytogel agar

Table 2.3 Type of media used in earlier works

? : No data given by the authors

2.3.11. Use of Activated charcoal (AC)

In this study activated charcoal (AC) is used as an additive to the media.

The influence of AC on culture media and plant propagation and its ability to regenerate several plant species has been previously reported (Thomas, 2008). The usage of AC in plant tissue culture is to prevent the development of abnormal plantlets, to enhance somatic embryogenesis, to increase shoot formation, plant recovery and rooting (Thomas, 2008).

Charcoal has the ability to absorb colloidal solids. The surface of AC contains specific areas ranging from 600 to 2000 m^2 g/l. Pore distribution varies from 10 µm to 500 µm (Thomas, 2008).

When AC is added to the medium, it prefers polar organics rather than non polar organics. Therefore, it has a great absorptive capacity towards aromatic products like phenolics and their oxidants, auxins including indole-3-acetic acid (IAA), naphthalene acetic acid (NAA), indoel-3-butyric acid (IBA) which is used in this study and cytokinins including BAP. But water soluble products including sugars like glucose, sorbitol, mannitol and inositol etc will not be removed from the medium or solution (Yam *et al*. 1990; Thomas, 2008).

The difficulty in using AC in this medium is that it may adsorb needed hormones (Ebert and Taylor, 1990; Ebert *et al*. 1993; Nissen & Sutter, 1990), vitamins (Weatherhead *et al*. 1978; Weatherhead *et al*. 1979; Pan and Van Staden, 1998), or metal ions such as Cu and Zn (Van *et al*. 2003).

2.3.11.1. Activated charcoal in plant tissue culture

A large number of reports are already published about the various application of AC in plant tissue culture and most of them confirmed that AC has a positive effect in medium promoting growth and development of plant tissues. Among the previous research (Loc *et al.* 2005) reported the effect of rooting on *Curcuma zedoaria*, (Thomas, 2008).

During micropropagation the leakage of phenol is very common and it often influences the result. Shoot induction from leaf explants of Exacum sp. had been reported of using the MS medium (Murashige and Skoog, 1962) supplemented with BA and NAA and the shoots were sub-cultured on modified MS (Murashige and Skoog, 1962) medium supplemented with PVP, 2iP, NAA, GA_3 and 0.06 g/l AC to simultaneously promote both elongation and rooting (Unda *et al.* 2007). In Eucalyptus sp. the shoots regenerated from Calli were sub-cultured to shoot elongation medium consists of MS medium (Murashige and Skoog, 1962) containing 1 g/l AC. (Barrueto *et al*. 1999).

Despite of these positive effects, the negative effect of AC in micropropagation was also reported; for example, it reduced the number of shoots in tissue growth (Komalavalli and Rao, 2000) or inhibited the shoot initiation (Tivarekar and Eapen, 2001) or suppressed bud sprouting from shoot nodes (Boggetti *et al*. 2001).

2.3.12. Growth regulators

There are natural chemicals produced within plant tissues with a regulatory, plus a nutritional role in growth and development. Generally, these compounds are active but at a very low concentrations. They are called plant growth substances (or, plant hormones). On the other hand we have synthetic chemicals which come with similar physiological properties. These compounds have an ability to modify plant growth by using some other means. These chemical compounds are usually considered as plant growth regulators. Some of the natural growth substances are prepared synthetically or through the fermentation processes and can be purchased from chemical suppliers.

There are several recognised classes of plant growth substance:

- Auxins,
- Cytokinins,
- Gibberellins,
- Ethylene,
- Abscisic acid.

Auxins and cytokinins are the most important for regulating growth and morphogenesis in plant tissue and organ cultures; in these classes, synthetic regulators have been discovered with a biological activity which equals or exceeds that of the equivalent growth substances (George, 1993).

IBA from Auxins and BAP from cytokinins (synthetic cytokinins) were used for this study.

IBA (Indole-3-butyric acid) with the molecular formula $C_{12}H_{13}NO_2$ and NAA (naphthalene acetic acid) with the molecular formula $C_{12}H_{10}O_2$ are among the auxins and BAP (6-Benzylaminopurine) with the formula $C_{12}H_{11}N_5$ from cytokinins are the

most favoured and common hormones employed in various experiments for tissue culture and micropropagation (Keng & Stanley, 2007; Loc *et al. 2005*; Miachir *et al. 2004*; George, 1993).

Auxins can promote the growth of plant tissues. Binding of auxin leads to lipid breakdown and acidification of the wall, increasing its extensibility and increasing the water potential of the cell; so water enters and cell expands.

Auxin also affects the mRNA transcription and they cause the cell to produce biologically active cell oligosaccharides. Auxins are able to remove genetically the original physiology of whole plant tissues which had previously determined their differentiated state; therefore auxin causes the cell to divide (George, 1993).

Auxins are generally used to stimulate callus production and cell growth in a culture medium or to initiate shoots, particularly roots, and to induce somatic embryogenesis and stimulate growth from shoot apices and shoot tip cultures (George, 1993).

The second group, cytokinins, can stimulate protein synthesis. The cytokinins are used to stimulate cell division in the culture medium and induce shoot formation or auxiliary shoot proliferation (George, 1993).

Based on previous works and several other papers reviewed, certain concentrations of both groups of hormones were tested and the best were chosen for the final action.

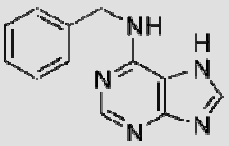
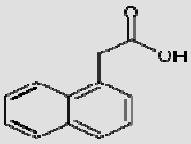
Growth regulators on different combination or concentrations ▶ / Previous works ▼	IBA  Indole-3-butyric acid	BAP  6-Benzylaminopurine	NAA  Naphthalene acetic acid
(Miachir *et al.* 2004) Plant : *Curcuma zedoaria Roscoe*	✖	0, 0.5,1.0, 1.5, 2.0 and 3.0 mg/l	BAP either alone or in combination with NAA: 0, 0.2, 0.5 and 1.0 mg/l
(Loc *et al*, 2005) Plant : *Curcuma zedoaria Roscoe*	0.5 mg/l	BAP either alone or in combination with IBA	3 mg/l, Rooted on → sub - culture; 2 mg/l
(Keng and Stanley, 2007) Plant : *Curcuma roscoe* and *Zingiber zerumbet*	Different combinations of IBA and BAP 0.5mg/l IBA + 0.5 mg/l BAP ↓ sub culture 0.5mg/l IBA + 0.5 mg/l BAP	✖	
This study	0.5,1, 1.5 mg/l IBA	combination of IBA and BAP 0.5 mg/l IBA + 3 mg/l BAP after various tests	2.5, 3,4 mg/l BAP ✖

Table 2.4 Growth regulators on different combination or concentrations earlier reports and this study ⟳: Combination, ⊢ : Rooted on

2.3.13. pH

Many plant cells and tissues in the *in vitro* system, can tolerate pH in the range of about 4.0-7.2; those inoculated into media adjusted to the pH between 2.5 to 3.0-8.0 will probably die (Butenk *et al*. 1984).

The pH range of 5.6-5.8 supports the growth of most meristem tips in culture. Slightly acid conditions usually give the best results. Base on previous experiments years ago until now, in a random sample of papers on micropropagation, the average initial pH studied for several different media is pH = 5.7, but adjustments have been made to as low as pH = 3.5 and as high as pH = 7.1 (Butenk *et al*. 1984; George, 1993).

2.3.14 The effect of autoclaving

Autoclaving changes the pH of media. If the media lacks the sugars, the change is usually small, unless the phosphate concentration is low. The media autoclaved with sucrose generally has a slightly lower pH compared to the one which is autoclaved without it, but if maltose, glucose, or fructose has been added instead of sucrose, the post- autoclave pH is significantly reduced (Owen *et al*. 1991).

The pH of liquid media containing MS (Murashige and Skoog, 1962) salts and sucrose, decreases based on previous works and experiments autoclaving from an adjusted level of 5.7 to 5.17 (Singila, 1984) to pH 5.5 (Owen *et al*. 1991), or pH 4.6 (Skirvin *et al.* 1986; George, 1993). Loc *et al.* (2005) used pH = 5.8.

2.4. Antibacterial assays

2.4.1. Extracts and solvents

Solvents are chemical compounds or mixtures (liquid, solid, or gas) that are mostly in liquid form at room temperature and pressure under the conditions of application and in which other substances (solid, liquid, or gaseous) can dissolve, and from which they can be recovered unchanged on removal of the solvent. So many substances conform to this definition; practically, all those that can be liquefied (Marcus, 1999).

Many classification schemes have been proposed for solvents, and a single major property, that may form the basis for the usefulness of solvents for certain applications, can often be employed in order to classify solvents. On the other hand, a few selected properties may advantageously be used to form the basis for the classification. Various solvent classification schemes have been presented and a common solvent classification scheme is:

(i) Non-polar solvents (such as petroleum ether and hexane) (Marcus, 1999),

(ii) Solvents of low polarity (such as chloroform) (Marcus, 1999),

(iii) Polar solvents (such as methanol, acetone) (Marcus, 1999)

(Wilson *et al*. 2005) used petroleum ether (60–80°C), n-hexane, chloroform, acetone and ethanol in their experiment while (Chen *et al*. 2008) used methanol as the solvents.

In this experiment, petroleum ether (40–60°C) with density d (20°C) = 0.642 - 0.656, chloroform ($M=138g/mol$) and methanol ($M=32.04g/mol$) were used as solvents.

2.4.2. Antibacterial Activity test

2.4.2.1 Antibacterial assay techniques

There are several various biological assay techniques which are used for measuring and monitoring the antimicrobial activity of natural compounds especially within plant extracts (José *et al.* 2006).

After reviewing previous related articles, it has become clear that there are two standard microbial techniques for studying the antibacterial activities, which have been used the most among the similar previous works so far; those two methods are considered as "agar-well diffusion method" and "filter paper disc method" and in some cases they both resemble. Wilson *et al.* (2005) and Niamsa & Sittiwet (2009) used the term "agar-well diffusion Method" while Chen *et al.* (2008) and Habsah *et al.* (2000) used the term "filter paper disc method" .

"Agar diffusion method" (disc-diffusion assay) was chosen for this study.

2.4.2.2. Agar-well diffusion Method

The term "agar-well diffusion method" comes from "the agar diffusion assay" devised in 1944 (Bauer *et al.* 1966). When the agar is turning into the molecules move through the matrix of the agar, this movement is actually called agar diffusion.

The level of the molecule's movement can be varied depending on the level of the molecule concentration; this must be under a controlled condition. This is the basis of the agar diffusion assay that is used to determine the susceptibility or resistance of a bacterial strain to an antibacterial agent, e.g. antibiotics.

In this method, the surface of the agar is occupied with the bacterial suspension which is first spread on the agar; then, antibiotic is applied to a number of wells on the plate. Different concentrations of a single antibiotic or a number of different antibiotics could be applied. Time is needed to allow the bacteria to grow. If bacterial growth is right up to the antibiotic containing well, then the bacterial strain is considered to be resistant to the antibiotic. If there is a clearing around the antibiotic, then the bacteria growth have been inhibited by the antibiotic. The size of the inhibition zone can be measured and related to standards, in order to determine whether the bacterial strain is sensitive to the antibiotic (Bauer *et al*. 1966; Wilson *et al*. 2005; Niamsa and Sittiwet 2009).

There is another way of performing this technique in which the discs of an absorbent material that have been soaked with the antibiotic of interest are placed directly onto the agar surface. After that, the antibiotic will diffuse into the agar. This version of agar diffusion is known as the Kirby-Bauer disc-diffusion assay (Bauer *et al.* 1966).

The agar diffusion assay technique allows bacteria to be screened in a routine, economical and time saving style for the detection of resistance.

More detailed analysis also can be done (Bauer *et al.* 1966; Wilson *et al*. 2005; Niamsa and Sittiwet 2009).

This is often used to determine the lowest concentration of an antimicrobial agent necessary to inhibit a microorganism after overnight incubation. This amount is known as the minimum inhibitory concentration (MIC) (Islam *et al.* 2008).

2.4.3. Bacterial Strains

There has been several species of bacteria used in different similar antibacterial activity experiments such as *Salmonella enterica, Staphylococcus aureus, Vibrio parahaemolyticus, E. coli,* (Chen *et al*. 2008), *Bacillus subtilis, Staphylococcus aureus, Micrococcus luteus, E.coli, Proteus mirabilis* and *Klebsiella pneumoniae* (Wilson *et al*. 2005).

Bacteria such as *Staphylococcus aureus, Staphylococcus epidermidis, Micrococcus luteus, Bacillus subtilis, Lactobacillus plantarum, E.coli, Salmonella typhimurium, Klebsiella pneumoniae, Proteus vulgaris, Pseudomonas aeruginosa* (Niamsa and Sittiwet, 2009) have been used on various extracts such as petroleum ether extract, hexane extract, chloroform extract, acetone extract, ethanol extract, water extract and methanolic extract. Wilson *et al.* (2005) chose *Bacillus subtilis* among the gram positive bacteria on petroleum ether extract, hexane extract, chloroform extract, acetone extract, ethanol extract and water extract and the antibacterial activity of *Curcuma zedoaria* was recorded on all except from the water extract. On the other side, Chen *et al.* (2008) did not perform any experiment on *Bacillus subtilis.* Wilson *et al*. (2005) and Chen *et al.* (2008) both did not get any results after performing the test on the gram positive *Staphylococcus aureus w*ith any type of extracts. Chen *et al*. (2008) found *Curcuma zedoaria* extracts effective on methanolic extract while Wilson *et al.* (2005), did not get any positive results. Wilson *et al*. (2005) found that all types of the extracts were effective on *Proteus mirabilis* and *Klebsiella pneumoniae* (except water extract) while Chen *et al* (2008), did not perform any test on said bacteria.

A comparison was made between the results (as antibacterial activity of *Curcuma zedoaria* on different types of extracts) of two previous studies (Wilson *et al*. 2005; Chen *et al*. 2008) in table 2.5.

	Bacteria on various extracts	Wilson *et al.* (2005)	Chen *et al.* (2008)
Gram positive	*Bacillus subtilis* on Petroleum ether extract Hexane extract Chloroform extract Acetone extract Ethanol extract Water extract	 Effective Effective Effective Effective Effective No effect	*No experiments done*
	Staphylococcus aureus on Petroleum ether extract Hexane extract Chloroform extract Acetone extract Ethanol extract Water extract	No effect on any type of extracts	No effect
	Micrococcus luteus on Petroleum ether extract Hexane extract Chloroform extract Acetone extract Ethanol extract Water extract	 Effective Effective Effective Effective Effective No effect	*No experiments done*
Gram negative	*E. coli* on Petroleum ether extract Hexane extract Chloroform extract Acetone extract Ethanol extract Water extract Methanolic extract	No effect on any type of extracts	Effective on methanolic extract
	Proteus mirabilis on Petroleum ether extract Hexane extract Chloroform extract Acetone extract Ethanol extract Water extract	 Effective Effective Effective Effective Effective No effect	*No experiments done*
	Klebsiella pneumoniae on Petroleum ether extract Hexane extract Chloroform extract Acetone extract Ethanol extract Water extract	 Effective Effective Effective Effective Effective No effect	
	Salmonella enterica on methanolic extract	*No experiments done*	Effective on methanolic extract
	Vibrio parahaemolyticus on Methanolic extract	*No experiments done*	No effect

Table 2.5 Comparing the results as "Antibacterial activity of *Curcuma zedoaria* "on different types of extracts in two earlier similar works .

2.4.3.1. *Staphylococcus aureus*

Spherical bacterium, *Staphylococcus aureus*, has been recognized as an important cause of disease worldwide for decades. It has become a major pathogen causing hospitals and the community acquired infections (Ursic *et al*. 2008).

Staphylococcus aureus is a well-established bacterial pathogen that can cause soft-tissue infections, skin infections, infections in immuno-compromised hosts, surgical patients and in those with indwelling medical devices (Kintarak *et al*. 2004; Schaffera and Lee, 2008), bacteraemia with metastatic complications, such as pneumonia, endocarditis, septic arthritis and osteomyelitis. *Staphylococcus aureus* is also a common wound infecting organism which can interact with cells via the extra cellular matrix protein fibronectin (FN) (Kintarak *et al*. 2004)

Meticillin-resistant *Staphylococcus aureus* (MRSA) strains which are very common in hospitals are responsible for nosocomial and community-acquired staphylococcal infections and major cause of nosocomial infection and many of these isolates are multidrug resistant (Schaffera and Lee, 2008).

MRSA poses a growing threat to public health world wide (Arya *et al*. 2004).

Staphylococci are an important cause of infection associated with indwelling medical devices (Baldassarri *et al*. 2001).

Staphylococcus aureus is a leading cause of both community- acquired and nosocomial bacteremia. Bacteremia with *Staphylococcus aureus* is associated with high morbidity and mortality, partly due to secondary complications such as endocarditis or hematogenic spread to other structures (Tkenheuer *et al.* 2004).

2.4.3.2. *Bacillus cereus*

Bacillus cereus is a common soil saprophyte (Granum and Lund, 1997), ubiquitous Gram-positive, spore-forming, motile and aerobic rod bacteria that also grows well anaerobically. It has been regarded as rather apathogenic or as an opportunistic pathogen (Kotirantaa *et al.* 2000; Granum and Lund, 1997), which is easily spread to many types of foods, especially of plant origin, but is also frequently isolated from meat, eggs and dairy products (Granum and Lund, 1997).

Bacillus cereus has been known to cause a variety of infections, including: endophthalmitis, bacteremia, septicemia, endocarditis, salpingitis, cutaenous infections, pneumonia and meningitis (Rasko *et al. 2005*).

Bacillus cereus is found as a contaminant in many food products causing two different types of food poisoning, the diarrhoeal type and the emetic type.

Complex enterotoxins which are produced during vegetative growth cause the diarrhoeal diseases which occur in small intestine. The emetic toxin which is produced by growing cells in the food causes the emetic type of poisoning (Granum and Lund, 1997).

2.4.3.3. *E.coli*

Escherichia coli is a Gram-negative bacterium from the family Enterobacteriaceae. The species is a normal intestinal flora of humans and domestic animals (Naylora *et al*. 2005). Most *E. coli* strains are harmless, but some, such as serotype O157:H7 can cause food poisoning in humans (Yokoigawa *et al*. 1999).

E.coli can also cause several human diseases such as mild diarrhoea, haemorrhagic colitis, hemolytic-uraemic syndrome, and thrombotic thrombocytopenic purpura Pathogenic (García-Sánchez *et al.* 2007). *E. coli* strains have been recognised since the early 1900s (Naylora *et al*. 2005; García-Sánchez *et al.* 2007).

2.4.3.4. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is one the most important opportunistic pathogens in animals and humans with Gram-negative hospital infections. Bacteriological investigations in various hospitals shows that Pseudomonas spp. mainly *Pseudomonas aeruginosa*, are the most frequently isolated organisms from pus, sputum, blood and other clinical material of patients. Patients with cystic fibrosis (CF), or following surgery, trauma and thermal burn have the *Pseudomonas aeruginosa* complications.

It is difficult to overcome *Pseudomonas aeruginosa* infections due to its high intrinsic resistance to antibiotics. Factors like the low permeability of the major outer membrane (OM) porins and the presence of multiple drug efflux are associated with the mechanisms of drug resistance in this species (Stanislavskya and Lam, 1997)

2.4.4. Results in earlier reports and current study

After reviewing previous reports and experiments, I concluded to use some certain strains of bacteria. Choosing the bacteria strains was also limited because of the BSL (Bio safety level) of some bacteria strains or availability during the time of performing this study. As it's illustrated in table 2.6, there are some certain bacteria strains which some scientists have already reported about their results when working with them and the results were all negative; hence there was no need to repeat their works or some bacteria has never been tried before in any previous studies, therefore it was decided to focus on those types which are not yet employed or have been only reported in a few earlier reports.

Bacteria on various Extracts	Wilson *et al.* (2005)	Chen *et al.* (2008)	This study
Bacillus subtilis	*Positive results*	*No experiments done*	☞ *No experiments done*
Staphylococcus aureus	*Negative results*	*Negative results*	☞ *Positive results*
Bacillus cereus	*No experiments done*	*No experiments done*	☞ *Positive results*
Pseudomonas aeruginosa	*No experiments done*	*No experiments done*	☞ *Positive results*
Escherichia coli	*Negative results*	*Positive results*	☞ *Negative results*
Micrococcus luteus	*Positive results*	*No experiments done*	☞ *No experiments done*
Vibrio parahaemolyticus	*No experiments done*	*Negative results*	☞ *No experiments done*
Salmonella enterica	*No experiments done*	*Positive results*	☞ *No experiments done*
Micrococcus luteus	*Positive results*	*No experiments done*	☞ *No experiments done*

Table 2.6 An overall comparison on results base on the bacterial strains which have been used in earlier reports through different extracts of *Curcuma zedoaria* and this study

2.5. Overall review

An overall review on this study is illustrated in table 2.7; growth regulators, media types, solvent extracts, and bacteria selection employed in earlier reports and this study.

Aspects ↓ / Experiment →	This study	Previous works
Growth regulators	combination of IBA and BAP 0.5 mg/l IBA + 3 mg/l BAP After various tests	BAP either alone or in combination with NAA/IBA BAP either alone or in combination with IBA
Media type	MS medium + Vitamins + Sucrose + Activated Charcoal (AC) + Phytogel agar	MS medium + Nicotinic acid / Thiamine and Pyridoxine / Glycine/ Myoinositol /Sucrose /Coconut water (CW) / AC + agar
Solvent extracts	Petroleum ether extract Chloroform extract Ethanol extract	Petroleum ether extract /Hexane extract /Chloroform extract /Acetone extract / Ethanol extract / Water extract
Bacteria selection	*Bacillus cereus* *Staphylococcus aureus* *E. coli* *Pseudomonas aeruginosa*	*Bacillus subtilis* *Staphylococcus aureus* *Micrococcus luteus* *E. coli* *Proteus mirabilis* *Klebsiella pneumoniae* *Salmonella enterica* *Vibrio parahaemolyticus*

Table 2.7 An overall review on earlier reports and this study essentials