1.0 INTRODUCTION

1.1 History of antimicrobial resistance

In the twentieth century, the discovery and application of the antimicrobial agents were the most significant achievements in the medical arena. Participants who had taken part in antimicrobial discoveries and had received Nobel prizes for their valuable works were Paul Ehrlich, Gerhard Domagk, Sir Alexander Fleming, Howard Florey, Edward P. Abraham, Ernst Boris Chain, and Selman Waksman (Owens & Lautenbach, 2008). During this time antimicrobial infections ceased to cause concern. However, microbial infections became problematic very quickly and led the microbial world to respond by developing different forms of antimicrobial resistance mechanisms against numerous antimicrobial drugs (Tenover, 2006). The first documentation of the emergence of antimicrobial resistance was in the use of optochin for the treatment of pneumococcal pneumonia. In 1939, β-lactamase was described by Abraham and Chain, as causing a worldwide emergence of resistance to penicillin in *Staphylococcus aureus* (Owens & Lautenbach, 2008). In the 1960's, the problem of β-lactamase production of virulent strains of *S. aureus* was treated successfully with methicillin. However, the first case of community-acquired (CA) methicillin-resistant *Staphylococcus aureus* (MRSA) was reported in 1961 as a serious nosocomial problem with high morbidity and mortality rates (Boucher & Corey, 2008; Moise & Sakoulas, 2008). Antimicrobial resistance is a growing worldwide problem, and the trend of the resistant bacteria either Gram negative or Gram positive pathogens is rising (Bhavnani & Tillostson, 2008). Specifically, staphylococci, enterococci, *Klebsiella pneumoniae*, and *Pseudomonas spp*, are the most prevalent organisms found in healthcare institutions, and in the communities (Tenover, 2006).

The existence of the resistant strains was a result of selective pressure of using antibacterial agents (Tenover, 2006). Also, a study has been reported which shows that

the development of antimicrobial resistance is usually the result of selective pressure which permits only the least susceptible bacteria populations to maintain their growth (Sheldon, 2007). The uses of antimicrobial agents for the long term could improve the development of slow resistance (Tenover, 2006). Many studies indicated there is a relationship between antimicrobial use and antimicrobial resistance, and the main factor behind the development of microbial resistance was the carelessness in the use of the antimicrobial drugs (Bronzwaer, 2002). In fact, antimicrobial resistance is a social problem as a patient who develops resistance to antimicrobial drugs could transmit the new resistant organisms to another who has never been exposed to the drugs. The adverse effects of antimicrobial resistance include contrary treatment outcomes, increased treatment costs, complicated hospital management, and increased risk of developing resistant infections in a healthcare setting (Scott & Roberts, 2008).

1.2 The need for the development of novel anti-MRSA agents

Staphylococcus aureus has been identified as an extremely successful human pathogen as well as a nosocomial pathogen since the emergence of resistance to methicillin (Francis et al., 2005). Enterotoxins, cytolytic toxins and cellular components of *Staphylococcus aureus* have been investigated and known as a virulence factor which causes severe diseases in human beings (Larsen & Mahon, 1995). In the 1960‘s, the first prevalence of MRSA was reported in a European hospital (Akinyemi, et al., 2005), and was also recognised in 1961 as a common cause for 30% to 40% of hospital-acquired infections (Francis et al., 2005). Strains of *S. aureus,* expressed multiple resistances to antibacterial drugs including gentimycin and methicillin as well, they were increasingly accountable for several infections in the United States and UK during the late 1970‘s (Akinyemi et al., 2005). The evolution of methicillin-resistant strains of *Staphylococcus aureus* was considered by clinicians as a main clinical and epidemiological pathogen in

hospitalized patients by the 1980‘s and emerged among patients who have gone through an artificial heart valve surgery as well (Larsen & Mahon, 1995). Also, it has infected intensive care unit admission ICU patients and repeated hospitalization and who are elderly (Moise & Sakoulas, 2008; Zuo et al., 2008). In fact, infection due to *S. aureus* also requires a high and increasing burden on health care resources, increasing morbidity and mortality (Nascimento et al., 2000; Adwan, et al 2008). In 2005, several statistical data estimated that the number of hospitalized American patients’ death by MRSA infections was approximately 19,000. Therefore, greater than 60% of *S. aureus* isolated from US hospital has been accounted as MRSA (Boucher & Corey, 2008). Likewise, outbreaks of MRSA are increasingly responsible for one fifth of all hospital-acquired infections, which according to the UK National Health Service has amounted to a cost of approximately £1 billion per year (Pesewu et al., 2008)

More than a decade ago, strains of MRSA have evolved since they differ from those strains seen in hospitals known as community-acquired MRSA (CA-MRSA). Investigations found CA-MRSA emerged from the community rather than hospitals, and these strains were identified and characterized by the presence of leukocidin as a virulence factor, and production of the Panton Valentine leukocidin. These pathogens expressed no multiple resistances mechanisms, and can be treated by using a single antibacterial drug while nosocomial MRSA infections are multidrug resistant (Munckhof et al., 2004). CA-MRSA has the ability to adapt and develop resistance easily as nosocomial MRSA. (Francis et al., 2005). Several studies illustrated that community-associated MRSA composed 8% to 20% of all MRSA isolates (Boucher & Corey, 2008).

Therefore, the prevalence of these pathogens CO-MRSA is a growing concern in the community. It has been found to be associated with soft tissue. This is evident in the skin infections which were reported in 34 individuals and 235 military recruits in

Alaska and Virginia, respectively. It can also cause necrotizing, severe pneumonia especially after influenza (Boucher & Corey, 2008). Infections of this kind in children, and young healthy adults were reported in France (Francis et al., 2005).

Many multi-sensitive CA-MRSA strains were first isolated in 2006, with the increasing global concern, Sam and his colleagues, (2008), reported nine clinical isolates of CA-MRSA in the University Malaya Medical Centre, in Malaysia for the first time. These pathogens, which express resistance to erythromycin, gentamicin, and ciprofloxacin, cause skin and soft-tissue infections. In general, Bacteria have successfully expressed resistance to various therapeutic agents, and transmit their resistance genes to their offsprings during their replication (WHO, 2001; Adwan et al., 2008).

MRSA is multidrug resistant to multiple therapeutic agents and the control of these bacterial infections can be quite challenging (Abu-Shanab et al., 2006; Moise & Sakoulas, 2008). However, it is sensitive to glycopeptides, i.e. vancomycin. As a result, it is the most effective antibiotic and the first choice for the treatment of infections, i.e. endocardities caused by MRSA (Larsen & Mahon, 1995; Moise & Sakoulas, 2008; Zuo et al., 2008). However, there has been global concern about development of resistance to vancomycin by MRSA strains. In 1996, strains of MRSA were isolated from Japanese patients, which exhibited less sensitivity after long-term vancomycin therapy (Hiramatsu, 2001). This was followed by isolation of more vancomycin-resistant *S. aureus* (VRSA) and hetero-VRSA as well (also known as vancomycin-intermediate *S. aureus* or VISA). For instance, The initial VRSA strain was isolated in 2002 in the USA and subsequent to this in New York in 2004, as well as in France, Korea, South Africa, and Brazil (Hiramatsu, 2001; Tenover, 2006; Bell, et al 2009). The prevalence of those strains has caused global concern worldwide. The effective way to reduce the selective pressure that helps the emergence of resistant organisms is by using antimicrobial

agents wisely (Tenover, 2006), i.e. vancomycin antibiotic should be used to treat infections caused by MRSA only to avoid loss of its activity (Assadullah et al., 2003). Although, vancomycin is considered as the more potent anti MRSA agent and the first choice for the treatment of nosocomial MRSA pneumonia (Hiramatsu, 2001), there are several side effects associated with its usage. For example, it enhances the toxicity of aminoglycosides, it requires drug serum concentration monitoring, penetration of poor lung tissue, and patients who have been treated with vancomycin have a significantly higher mortality rate when afflicted with MRSA pneumonia (Moise & Sakoulas, 2008).

The outbreaks of antibiotic resistance is an issue of growing public concern (Nascimento et al., 2000). Also, the growing threat of MRSA has led to the development of novel anti-MRSA agents with different mechanism of action (Zuo et al., 2008). In 2001, World Health Organization issued strategies to battle and suppresses the global problem and one of the recommended strategies was the development of new drugs and vaccines (Tillotson, 2008).

However, the incidence of undesirable effects that were associated with certain new antimicrobial agents lead the World Health Organization in 2002, to discover new antimicrobial drugs particularly from medicinal plants (Zaidan et al., 2005), as well as a significant source for a variety of drugs (Nascimento et al., 2000).

1.3 The therapeutic value of medicinal plants in drug discovery

Various kinds of sources such as soil, animal, microorganism, and plants have been studied to discover novel antimicrobial compounds (Nitta et al., 2002). Plants have been known for a long period of time as a value source of natural products and have been used due to their antimicrobial activity for the treatment of infectious diseases and to maintain human health (Nascimento et al., 2000).

Hundred years ago, plants were considered to consist of a bioactive compound which could be used as a remedy for various diseases caused by pathogenic bacteria, i.e. plants extract from western North America, has been found to possess therapeutic activity for human immunodeficiency virus-1 reverse transcriptase. Similarly, crude extract of *Shorea hemslyana* and *Cyphostemma bainessi* showed great anti MRSA activity which significantly reduced the number of viable cell (Nitta et al., 2002). Likewise, in the Akwapim-North district of Ghana, they used plants in folk medicine to treat bacterial and other skin disorders (Pesewu et al., 2008). Furthermore, from ancient time Nigerians have utilized the curative potential of plants to treat several ailments. Trinidad and Bahamas has also used *T. africana* leaves as an effective therapy in lowering blood pressure, stomach upsets and gastro intestinal infections (Ogbonnia et al., 2008). In addition to this, for a long time the use of herbal medicines has been incorporated into primary health care in countries such as China (Akinyemi, et al., 2005; WHO, 2002). India which is endowed with over 20,000 medicinal plant species has also been known to use a large number of formulations in folk medicine familiar to its rural communities (Verma & Singh, 2008).

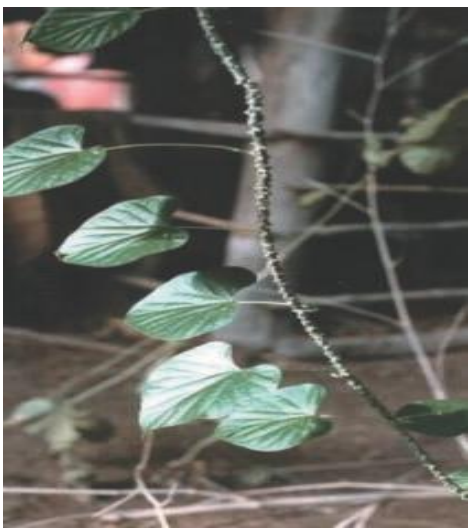
As a result of the prevalence of microbial resistance against large varieties of antibiotics (Akinyemi et al., 2005), several new techniques of isolation and characterization combined with development of new pharmacological method have led to interest in medicinal plants as antiseptics and antimicrobial agents in dermatology (Weckesser et al., 2007). It has also enhanced economics and social benefits (Zaidan et al., 2005). Thus, further investigation of plants should be carried out to understand their properties, safety and efficiency (Nascimento et al., 2000). The World Health Organization has also recognized the use of herbal medicine in their Traditional Medicine Strategy 2002-2005. Various international organizations, such as the Association for the Promotion of Traditional Medicine, and the Islamic Organization for

Medical Sciences, are striving to promote production of herbal medicine ensure their safety and efficacy (WHO, 2002).

1.4 *Tinosprora crispa* L

Tinospora crispa which belongs to the Menispernaceae family is known by many different names like *crispum Linn*., *Menispermum rimosum Blanco* and *Menispermum tuberculatum*. In certain instance it is known by its local name such as *bratawali,* or *andawal in Indodesia* (Dweck & Cavin, 2006). Likewise in peninsular Malaysia it is referred to as *'Akar patawali'* (Sulaiman et al., 2008) and *' Sapai'* in Sabah (Ahmad & Ismail, 2003).

In terms of appearance, *Tinospora crispa* can be likened to a woody and glabrous climber decked with shiny green leaves (Dweck & Cavin, 2006). It is very easy to distinguish its young stems from the old ones as they take on a smooth texture while old steams are distinguishably tuberculate and have extremely bitter sap (Chavalittumrong et al., 1997).



Pictures adapted from (Dweck & Cavin, 2006)

Figure 1: pictures of *Tinosprora crispa* plant showing branches, steams, and leaves.

1.4.1 Traditional use of *Tinosprora crispa*

Traditionally, *T. crispa* has been widely employed as a remedy for many afflictions such as fever, hyperglycemia, intestinal worms, wounds, and skin infection by Malaysian, Indonesian, and Thailand people (Sulaiman et al., 2008). In Sabah, it has been used for hypertension (Ahmad & Ismail, 2003) treating diabetes, and lumbago (Dweck & Cavin, 2006). Furthermore, *T. crispa* was known to treat Malaria fever in Vietnam, and has been used to treat tropical ulcer and rheumatism in Philippine (Dweck & Cavin, 2006). Also, it is an effective remedy for stimulation of an appetite enhancer (Sartori & Swift, 2003; Zulkhairi et al., 2008), as protection from mosquito bites (Zulkhairi et al., 2008), and as effective cure for treating tooth, coughs, asthma, pleurisy, and stomach ache, too (Sulaiman et al., 2008).

1.4.2 Previous study on *Tinospora crispa*

Many scientific studies indicated that the extract of *T. crispa* has been exhibited antimalarial (Rahman et al., 1999), antifilarial effects, and as contributor involved in pain and inflammation processes, because of its restriction of the nitric oxide synthesis and its release. In addition, Sulaiman et al. (2008) have further investigated two activities, antinociceptive and anti-inflammatory of the steams of *T. crispa*. Also, it has been found to have antibacterial (Zakaria et al., 2006), antipyretic, cardiotonic effects (Kongkathip et al., 2002), antihypoglycaemic (Noor & Ashcroft, 1989; Pannangpetch et al., 2006), and insulinotropic effects in experimental animal (Noor & Ashcroft, 1998). Past studies have, in fact, recorded that at certain dose of the ethanol extract could decrease carrageenan- induced hind paw edema, whereas aqueous extract reduced fever in Wistar rates at certain dose, too (Chavalittumrong et al., 1997). Zulkhairi et al., (2008) have investigated that the water crude extract of the steam of *T. crispa* possess has been found to be an effective source of natural antioxidants and nutrients as well as

a moderate anti-proliferative which effect on selected human cancer cell lines.

1.4.3 Chemical components isolated from *Tinosprora crispa*

The proximate analysis of *T. crispa* steams and leaves showed that *T. crispa* consists of certain nutrients and minerals, protein, fat, carbohydrate, ash, moisture, fiber, and energy (Zulkhairi et al., 2008). Beside this, many studies and reported cases have shown that several chemical compounds previously isolated from the *T. crispa* steam include bergenin which is known as an antioxidant and free radical scavenging agent, secoisolariciresinol, and flavonoids (apigenin) known for its ability to act as a powerful anti-oxidants, anti-allergic, and antiviral properties (Chavalittumrong et al., 1997). Other compounds that have also been found include borapetol A and B, borapetoside A and B, tinocrisposide, *N*-formylanondine, *N*-formylnornuciferine, secoisolariciresinol, *N*-acetyl nornuciferine, γ-sitosterol, picroteine, and tinotubride, and quaternary alkaloids (Chavalittumrong, et al., 1997; Sulaiman, et al., 2008; Zulkhairi & Abdah, 2008). Furthermore, Kongkathip and his coleagues (2002), have also isolated for the first time two new triterpenes, cycloeucalenol, and cycloeucalenone from *T. crispa* steams, while tinotufolin C, D, E, and F was isolated from fresh leaves (Chavalittumrong et al., 1997). Amongst the chemical constitutes the most significant compound of the above is the alkaloids which can interfere with microtubule function. It also has been established to have anti-cancer properties, and as well as used for treating numerous solid tumours by combination with chemotherapy regimens (Zulkhairi et al., 2008).

1.5 Antimicrobial susceptibility test methods

1.5.1 Disc diffusion test

Disk diffusion testing is commonly known as Kirby-Bauer. It is mainly used to test non-fastidious, rapidly growing bacteria (e.g., *Staphylococcus*). The test has been altered; hence, it is suitable for some fastidious organisms (e.g., *Haemophilus spp*). In this test, blank or paper discs impregnated with antimicrobial drugs or extract of some plants are placed on the inoculated surface of Muller Hinton agar. Once contact with the agar, the antimicrobial agents or plant extract that has antibacterial properties starts to diffuse out the disc and into the agar in a radial pattern. The nearer to the disc the more the drug is concentrated and the further away from the disc, the lower the concentration of the drug. If the test organism was inhibited by the concentration of drug, a radial zone of inhibition around the disc is formed. If the growth of test organism was not inhibited by the drug, a lawn of growth is formed (Jorgensen & Turnidge, 2007).

There are many advantages of using disc diffusion testing; these include its simplicity, inexpensiveness, flexibility, and ability to test several different antibiotics at the same time. However, the limitation of the disc diffusion test is, it would not work well if the test drug or test compound is unable to diffuse well through the agar. The size of inhibition depends on the susceptibility of the test organisms to the drug, and agar depth. In addition, a potential drawback of using disc diffusion testing is that its results are qualitative. Thus, quantitative result showing the susceptibility level in some cases may be required. (Hindler & Jorgensen, 2007; Jorgensen & Turnidge, 2007)

1.5.2 Dilution methods: Broth dilution

Broth dilution is one of the dilution testing. It is a well standardized and reliable method that can be carried out for determination of the minimum inhibitory (MIC) value. The MIC value is defined as the lowest concentration of a test substance that can completely inhibit the growth of the organism. These concentrations are generated using double fold serial dilution of the substance and allow for evaluating the relative degree of susceptibility of an organism to the test substance as well as comparing the activity of the test substance against various organisms (Jorgensen & Turnidge, 2007).

1.6 Acute oral toxicity testing

The use of herbal medicine in developing countries is more common. Herbal remedies are often thought to be safe because they are natural. However, these products may contain active compounds that could cause some form of toxicity or adverse effects. Hilaly et al., 2004; D´eciga-Campos et al., 2007; Obici et al., 2008 have illustrated that many plants have shown to cause significant cytotoxicity, neurotoxicity, mutagenecity, carcinogenecity or embryotoxicity. Poisonous plants can be found ubiquitously although the common use of traditional remedies. Therefore, preclinical toxicological studies of plant compound should be investigated, to ensure their security, efficacy, and quality for human consumption.

In the current investigation, a toxicity study of the crude extracts of *T. crispa* was conducted according to the acute oral toxicity fixed-dose procedure proposed by the international guidelines of Organization for Economic Cooperation and Development (OECD, 2001). Acute toxicity can be defined as “adverse effects occurring following oral or dermal administration of a single dose of a substance, or multiple doses given within 24 hours” (OECD, 2001). The main focus of performing an acute toxicity test is to obtain information about the biological activity and its mechanism (Walum, 1998).

The time frame for an acute toxicity may change accordingly to experimental requirements, and animals are usually continuously observed upon administration of the test substance up to 14 days or longer.

According to (OECD, 2001) the median lethal oral dose, or LD_{50}, is defined as ''the statistically derived single dose of a substance that, when administered in an acute toxicity test is expected to cause death in 50 percent of the treated animals in a given period.'' For the objective of harmonizing health hazard in humans, chemical labelling and classification of acute systemic toxicity based on oral or dermal LD_{50} values has been allocated by (GHS, 2005) to 5 toxicity categories. The first category being the most toxic and the last one least toxic, the categories as follow: <5 mg/kg body weight,>5< 50mg/kg, > 50< 300mg/kg, >300< 2000mg/kg, and >2000< 5000. Thus, the value of LD_{50} was estimated as equal to the administrated dose if it is equal to 50 percent mortality. The value of LD_{50} was also estimated as greater than administrated dose if less than 50% mortality and vice versa (Douds, 1997).

1.7 Objectives of the study

1. To screen antibacterial activity in aqueous and ethanol extracts of *Tinospora crispa* against MRSA.
2. To determine the minimum inhibition concentration value of *Tinospora crispa* to susceptible bacterial strains.
3. To examine for acute toxicity of orally administrated *Tinospora crispa* extracts suspension.