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).

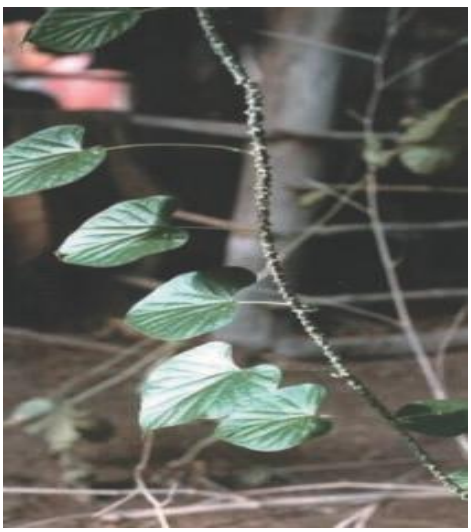
As a result of the prevalence of microbial resistance against large varieties of antibiotics (Akinyemi et al., 2005), several new techniques of isolation and characterization combined with development of new pharmacological method have led to interest in medicinal plants as antiseptics and antimicrobial 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.

2.0 MATERIALS AND METHODS

2.1 Plant material and extract preparation

In the current study, aqueous and ethanolic extracts were derived from *Tinospora crispa* plant. The *T. crispa* plant in dried form was kindly provided by Assoc. Prof. Dr. Mahmood from the Immunology Laboratory of University of Malaya.

2.1.1 Aqueous extracts preparation

The dried form of plant was mixed with sterile distilled water in a ration of 1:20 (100 g in 1 L solvent), and was stirred and heated for about 4 hours. After cooling, the extract was filtered by using Whatman No.1 filter paper. The filtrate was collected and frozen in ice cube container. The frozen ice cube was freeze-dried (i.e. lyophilisation) to obtain concentrated, aqueous extracts in powder form.

2.1.2 Ethanol extracts preparation

The dried plant material was mixed and macerated with absolute ethanol at a 1:20 ratio (100 g in 1 L solvent) for 7 days. Then the extract was filtrated through Whatman No 1 filter paper and then followed by rotor- evaporated the supernatant by using the BUCHI Switzerland Rotary Evaporator to remove the ethanol and to obtain concentrated, oily extract. The crude extracts were then kept at -20 ºC in sterile universal bottles.

2.1.3 Sterility proofing of the extracts

With reference to tests done by Sule & Agbabiaka (2008), we made some modifications to the sterility proofing of the extracts by introducing 2ml of the extract into 10 ml of Muller Hinton broth, and incubated at 37 ^{0}C for 24 hours. The absence of

turbidity or clearness of the broth after the period of incubation signifies the presence of a sterile extract

2.2 Identification of bacterial strains

The eight Methicillin resistant *Staphylococcus aureus* (MRSA) pure isolates used in this study were kindly provided by Assoc. Prof. Dr. Yassim from the Microbiology Laboratory of University Malaya Medical Centre and a reference strain *Staphylococcus aureus* ATCC 25923 were obtained from the Molecular Bacteriology Laboratory, Faculty of Medicine. All samples were cultured and sub-cultured again for purity on Columbia Horse Blood Agar plates (Biomedia Laboratories Sdn. Bhd.). Colony morphology and Gram staining was carried out to confirm the identity of working strains as mentioned in the Textbook of Diagnostic Microbiology (Hindler & Jorgensen, 2007).

2.3 Antimicrobial susceptibility testing

2.3.1 Disc diffusion test

Disc diffusion method for antimicrobial susceptibility testing was carried out based on recommendations given by the Clinical Laboratory Standards Institute, CLSI (Hindler & Jorgensen, 2007; Jorgensen & Turnidge, 2007).

2.3.2 Preparation of impregnated discs

A stock solution of each plant extracts was prepared by dissolving 100 mg of extract with one ml of their respective solvents (sterile distilled water and 99.9% dimethyl sulfoxide. Ten, 30 µl, 50 µl of a final concentration of 100 mg/ml have then used to impregnate in sterilized 6 mm blank discs (Oxide, UK). Distilled water and

dimethyl sulfoxide-loaded discs were used as negative controls for aqueous and ethanolic extracts respectively. All impregnated discs were ensured to be fully dried in 45 ºC incubator for 18 to 24 prior to the application on bacterial lawn (Zaidan et al., 2005). The standard antibiotic disc used as positive controls was vancomycin (30 μg; Becton-Dickinson, USA) for all *S. aureus* strains.

2.3.3 Inoculums and inoculation procedure

The inoculum density was standardized to achieve a final concentration of 1.5 x 10^8 CFU/ml by the growth method. Three to five single colonies from an agar plate culture were suspended in four to five ml of Mueller Hinton broth and incubated at 37 ºC until visibly turbid (0.5 McFarland standard) (Jorgensen & Turnidge, 2007). Furthermore, the inoculum suspension was used within 15 minutes of standardization, which is a very important factor to avoid any change of the size of inoculums or lose their viability (Wanger, 2007). A sterile cotton swab was dipped into the standardized bacterial inoculum suspension, and then it was streaked over the whole dried surface of 90 mm Mueller-Hinton agar (MHA; Becton-Dickinson, USA) plates twice. The agar plate was rotated about 60 degrees each time to ensure that the inoculum was distributed the entire agar surface. In order to expel the excess moisture from the inoculated plates their lids were left ajar for less than 15 minutes.

2.3.4 Application of impregnated discs

The discs which had been impregnated with plant extracts using sterile forceps were applied on the inoculated Mueller Hinton agar once it has completely dried. The disks were pressed gently to ensure uniform contact with agar surface. Furthermore, each one of the test plates was comprised of no more than five discs which placed about equidistance to each other to avoid the overlapping of inhibition zone. Three treated

discs, one positive control, which is a standard commercial antibiotic disc, and the last one negative control. Then, the plates were inverted and incubated for 24 hours at 37°C. The diameter of inhibition zone either around the treated discs or around the control discs were measured for the antibacterial activity assessment. If present, their diameters were measured to the nearest whole millimetre with a ruler. All tests were carried out three times to ensure the reliability, and the average of the three replicates for each extract, and antibiotic were calculated.

2.3.5 Minimum Inhibitory Concentration (MIC)

The highest dilution of the extracts that inhibit the growth (no visible bacterial growth when compared with control tube) but not kill the organism was defined as MIC. For the active plant extracts which showed inhibition zone in some test plate from the disc diffusion method were further tested to determine MIC values by broth macrodilution method based on recommendations given by the Clinical Laboratory Standards Institute, CLSI (Hindler & Jorgensen, 2007; Jorgensen & Turnidge, 2007).

Broth dilution procedure (macrodilution) was carried out for quantitative measurement to investigate *in vitro* the antimicrobial property of plant extract against the test bacterial isolate.

2.3.6 Preparation of extract dilutions

A stock solution of plant aqueous extract was prepared by dissolving 100 mg of extract in 1 ml of sterile distilled water. Likewise, for the ethanol extract, 100 mg of extract was dissolved in 1 ml of 10 % Tween-20 rather than the original solvent (i.e. Dimethyl sulfoxide), and so the initial concentration of the plant extract (100 mg/ml) was diluted using double fold serial dilution by transferring 1ml of the sterile plant extract (stock solution) into 1ml of sterile Mueller Hinton broth to obtain 50 mg/ml

concentration. The above process was repeated several times to obtain other dilutions: 25 mg/ml, 12.5 mg/ml, and 6.25 mg/ml 3.125 mg/ml 1.56 mg/ml, 0.78 mg/ml, 0.39 mg/ml, 0.2 mg/ml, and finally 0.1 mg/ml.

The concentrations were prepared to a volume of 0.5 ml in separate microcentrifuge tubes at double the intended concentrations so that addition of equal volumes of bacterial inocula in next steps would result in the desired final concentrations in each tube.

2.3.7 Inoculation procedure

The bacterial inoculum were prepared with Mueller-Hinton broth (MHB; Becton-Dickinson, USA) which was similar to the disc, then incubated at 37 ºC for 18-24 hours, and the bacterial concentration was adjusted to a 0.5 McFarland standard (1.5 x 10^8 CFU/ml). The suspension was then diluted 1:100 with sterile broth to obtain a cell number of approximately 10^6 CFU/ml. Next, 0.5 ml of the standardized bacterial suspension was then added to the tubes containing the previously prepared 0.5 ml of diluted extracts, resulting in a recommended final cell count of about 5 x 10^5 CFU/ml. A tube containing broth, extract solvent either distilled water or 10% (v/v) Tween-20, and the inoculums was known as positive growth control. On the other hand, a tube containing broth without inoculum, and extract solvent served as the negative control. All the tubes were incubated overnight at 37 ºC.

2.3.8. Determination of MIC values

In order to find out if there was any bacterial growth, the turbidity of the solution in each tube was observed on the next day. To ensure the presence or absence of bacterial growth in the tubes, a standard loop of the suspensions in each tube was inoculated on 3mm MHA and incubated overnight at 37 ºC. The plates were observed

following incubation to confirm absence or growth of bacteria. The lowest concentration of extract dilution showing no visible growth was recorded as the MIC value. The tubes were further incubated another 24 hours and plated again to observe for absence/growth after 48 hours incubation period. Likewise, the MIC value after 48 hours incubation was recorded too.

2.4 Antibacterial effects of several plant solvents

Several common plant solvents for the antibacterial susceptibility test were assessed before a particular solvent was chosen for the MIC assay as well as a medium to study the acute toxicity. The purpose of carrying out this investigation was to ensure that the chosen solvent did not contain antibacterial property that could interfere with the MIC assay. The plant solvents examined were sterile distilled water, DMSO, absolute ethanol, and 10% Tween-20, while the bacterial strains used were MRSA strains and *S. aureus* ATCC 25923.

The steps of this assay were performed by following the same procedure of the MIC assay. Five hundred µl of each solvent was added to five hundred µl standardized bacterial suspension that was adjusted similarly to the MIC assay (final cell count of approximately 5×10^5 CFU/ml). The tubes were incubated and then streaked onto MHA to observe the presence of bacterial growth.

2.5 Acute toxicity study

Adult male and female *Sprague Dawley* rats (8- 10 weeks old) were obtained from the Animal House, Faculty of Medicine, University of Malaya, Kuala Lumpur (Ethics Approved number: PM 07/05/2008 MAA (a) (R)). The rats which weighed between 180-200 g were given tap water and standard pellet diet *ad libitum*, for a minimum of five days before the start of the treatment to allow for acclimatization.

Furthermore, when the rats were conveyed to the Experimental Animal Unit, they were kept in separate cages.

2.5.1 Acute oral toxicity study

In order to determine a safe dosage for the plant extract (aqueous and ethanol extract) a study of the acute toxicity was undertaken. Thirty six *Sprague Dawley* rats (18 males and 18 females) were equally assigned into 3 groups for each extract labelled as a vehicle (10% Tween-20, 5 ml/kg); 2 g/kg and 4 g/kg of plant extract preparation, respectively. The amount of the plant extract dosage given to each rat was initially based on the calculated animal's body weight. (Douds, 1997). Prior to testing, all animals were fasted overnight and food was withheld for a further 3 to 4 hours after dosing. The acute oral toxicity study was carried out based on the OECD Guideline for Testing of Chemicals 420 (2001).

2.5.1.1 Mortality and behavioural observation

The animals were observed for 30 min and 2, 4, 8, 24 and 48 h after the administration for mortality or behavioural changes indicative of toxicity. Signs of mortality were continuously observed and twice daily for up to 14 days. The animals were fasted and sacrificed on the following day (15^{th} day), and subjected to necropsy for gross observation, liver, renal function tests, and histological examination.

2.5.1.2 Body weight analysis

The individual weights of each rat were recorded before administering the plant extract as well as on the day of termination. This was done in order to determine if there were any variances in the body weight of each rat as suggested by OECD guidelines.

2.5.1.3 Liver and renal function analysis

Prior to termination, all animals were fasted. On the day of the termination, the animals were anaesthetised with diethyl ether in a chamber. Blood from each rat was drawn from the jugular vein and collected in separate BD Vacutainer® blood collection tubes with clot activator (Becton-Dickinson, USA). All the samples were sent immediately to the Clinical Diagnostic Laboratory of the University Malaya Medical Centre for liver and renal function tests. The results were compared to their respective control groups for the following parameters: total protein, albumin, globulin, alanine aminotransferase (ALT), and aspartate aminotransferase (APT), alkaline phosphatise (AP), creatinine, and urea levels.

2.5.1.4 Gross necropsy

Gross necropsy was performed on all terminated rats after blood collection.

2.5.1.5 Histological examination

For histological examination, the livers and kidneys of each rat were then excised and fixed in the freshly made 10% neutral buffered formalin for 6 hours. After the fixation, all tissue from the organs collected were trimmed appropriately and kept in a cassette for overnight with 10 % buffered formalin. Then the tissue processed in an automated tissue machine to undergo dehydration, clearing and impregnation for 16 hours. The tissues were embedded in paraffin, sectioned by microtome and stained with haemotoxylin and eosin (H and E) stain. The slides were then observed and analyzed under the light microscope with magnification of x10, x40 and x100 (oil immersion). The purpose of the doing histology section is to observe for any sign of histopathological changes in the organs.

2.6 Statistical analysis

Data was expressed in mean ± S.E.M. Comparisons of body weights and liver function parameters were compared against the animals' respective vehicle groups using one-way analysis of variants (ANOVA) and Bonferroni's post hoc test with SPSS Statistics 18.0 software. The treatment group were significantly different when *p* value is less than 0.05.

3.0 RESULTS

3. 1 Bacteriological characteristic study

The cultural characteristics of the bacterial isolates on horse blood agar plates were observed after incubation period. The tested organisms were gray-white colour, entire margin, and small in size. The shape and texture were convex and shiny, respectively. In addition, all bacterial strains were subjected to Gram staining to ensure the identity of the test bacteria. When observed under microscope, the bacterial strains culture showed uniform arrangement of Gram-Positive Cocci occurred in clusters, pairs; long and short chains shape **(Figure 2).** Also, the bacterial strains showed beta haemolysis after overnight incubation at 37 ºC for 24 hours on Columbia Horse Blood Agar.

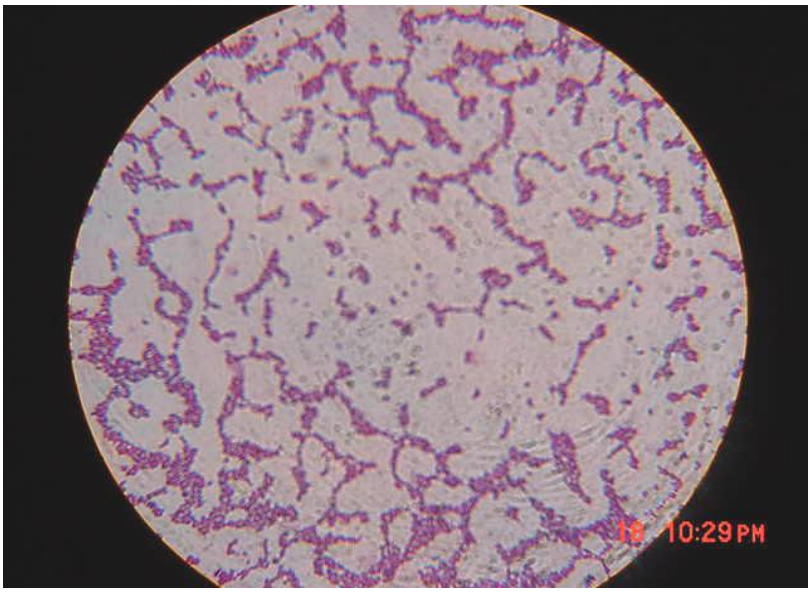


Figure 2: Gram stain of *S. aureus* bacteria from Columbia Horse Blood Agar (Magnification, 100x)

3.2 Antimicrobial susceptibility testing

3.2.1 Disc diffusion method

The results obtained from the disc diffusion method followed the same trend as the Minimum Inhibitory tests. The aqueous extracts of *Tinospora crispa* did not show any inhibition on all the tested organisms at the concentration used **(Figure 3).** On the other hand, using the same concentration, the ethanolic extracts of the plants inhibited the tested organisms at different rates **(Figure 4).** Hence, the ethanolic extracts of *Tinospora crispa* had antibacterial property on the tested organisms as compared to aqueous extract.

The appearance of zone inhibition that produced around the discs was observed and their diameters measured against a dark, non-reflective background. The zone diameter was measured in mm units. All the diameters of zone inhibition of the positive controls fall within susceptible ranges according to the interpretive standards for disc diffusion susceptibility testing by CLSI (Hindler & Jorgensen, 2007; Jorgensen & Turnidge, 2007), whereas all negative controls discs showed no zones of inhibition.

Figure 5 illustrates the mean diameter of zones of inhibitions obtained from the ethanolic extract of *T. crispa.* Ethanolic extract was found to show zones of inhibition against all MRSA strains, and *S. aureus* ATCC. All –ST/09 strains and *S. aureus* were tested at volume of 10, 30, 50 µl per disc of 100 mg/ml concentration of the extract. At 50 µl per disc of 100 mg/ml concentration of the extract, the highest volume used, the ethanolic extract gave mean diameters of zones that ranged from 11.3 mm (for *S. aureus* ATCC) to 13.5 mm (for MRSA ST/0904-30).

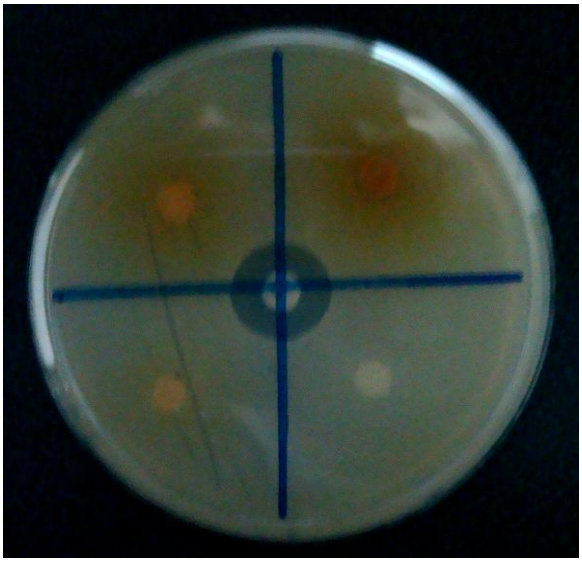


Figure 3: Plate showed the absence of any zone of inhibition by aqueous extract of *T. crispa*, and middle zone of inhibition given by vancomycin positive control.

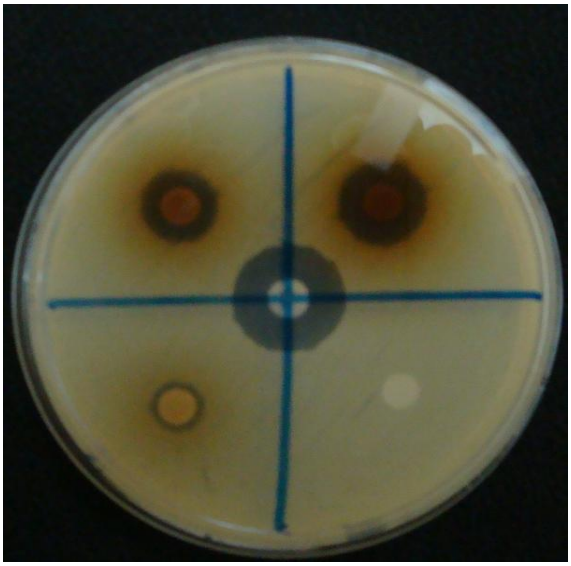


Figure 4: Plate showed clear, measureable inhibition zones of MRSA ST/0903-22 given by vancomycin positive control (middle zone) and discs loaded with different volumes of 100 mg/ml concentration of *T. crispa* ethanolic extract (top right: 50 µl; top left 30 µl; and bottom left: 10 µl of 100 mg/ml). The bottom right disc is the negative control.

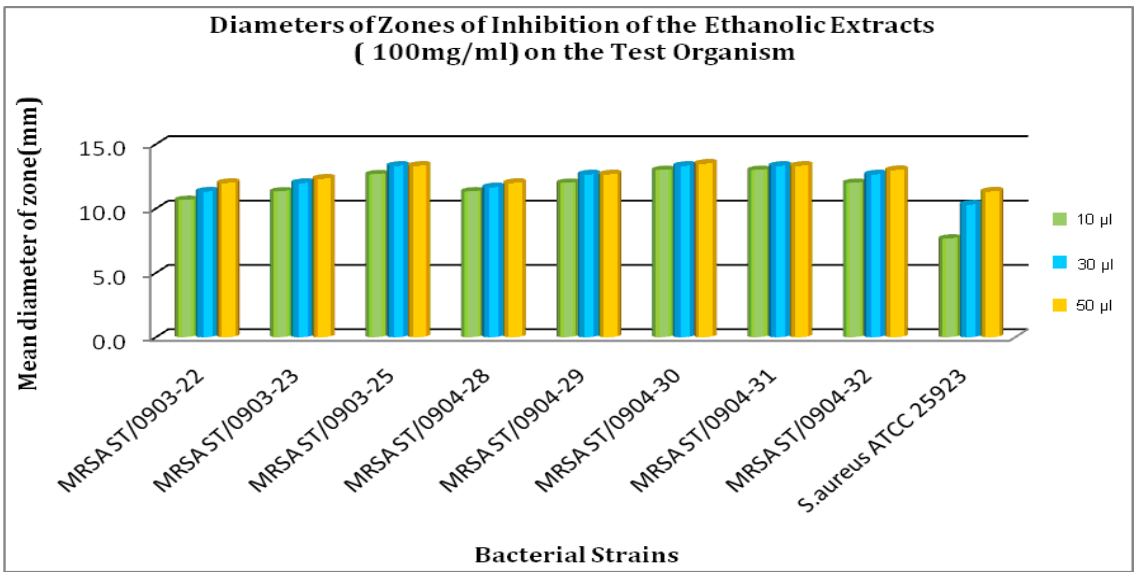


Figure 5: Mean diameters of zones of inhibitions where bacterial growth was inhibited by ethanol extract of *Tinospora crispa*

Mean values calculated from three replicate readings of the zones to the nearest millimetre (mm).

All –ST/09 strains denote MRSA strains. All tested at volume of 10 µl, 30 µl, and 50 µl per disc of 100 mg/ml concentration of the extract.

3.2.2 Minimum Inhibitory Concentration (MIC)

The bacterial strains showed measurable zones of inhibition to *Tinospora crispa* from the disc diffusion assay were further tested to determine their MIC values. Similarly, the extract which failed to inhibit the growth of bacteria was further tested to ensure that it has no antibacterial activity against tested organisms and to ensure the bacterial resistance as well. All MIC values obtained were tabulated in **(Table 1)** and a comparison of the results of each incubation time was analysed. From the results, the 48 hours incubation period gave lower MIC values in some strains for ethanol extract as shown in **(Figures 6).**

The result of the Minimum Inhibition Concentration (MIC) showed that the ethanolic extract exhibited varying inhibitory effects on the tested organisms. MRSA ST/0904-31 was the most susceptible strain to the ethanolic extract of *T. crispa*, unlike ST/0903-23, MRSA ST/0903-25 and MRSA ST/0904-32 which showed no variance for the two different periods (MIC 50.0 mg/ml). Furthermore, the extract inhibited the growth of four pure clinical isolate (MRSA ST/0903-22, MRSA ST/0904-28, MRSA ST/ 0904-29, MRSA ST/0904-30, MRSA ST/0904-31), and *S. aureus* ATCC 2592 with MIC 25.0 mg/ml after a 48 hour incubation period. **(Figure 6).**

In contrast, the results of the Minimum Inhibitory Concentration (MIC) showed that the aqueous extract of the plant failed to show inhibitory effect on majority of the test organisms at the test concentration used **(Table 1).**

Table 1: Minimum Inhibitory Concentration (MIC) of extract of *Tinospora crispa* on susceptible bacteria strain

Bacterial Name	24 hours [a]	48 hours [a]	24 hours [b]	48 hours [b]
MRSA ST/0903-22	50	25	-	-
MRSA ST/0903-23	50	50	-	-
MRSA ST/0903-25	50	50	-	-
MRSA ST/0904-28	50	25	-	-
MRSA ST/0904-29	50	25	-	-
MRSA ST/0904-30	50	25	-	-
MRSA ST/0904-31	25	25	-	-
MRSA ST/0904-32	50	50	-	-
S. aureus ATCC 25923	50	25	-	-

(-) No inhibition demonstrated,

[a] Ethanol extract of *T. crispa*

[b] Aqueous extract of *T. crispa*

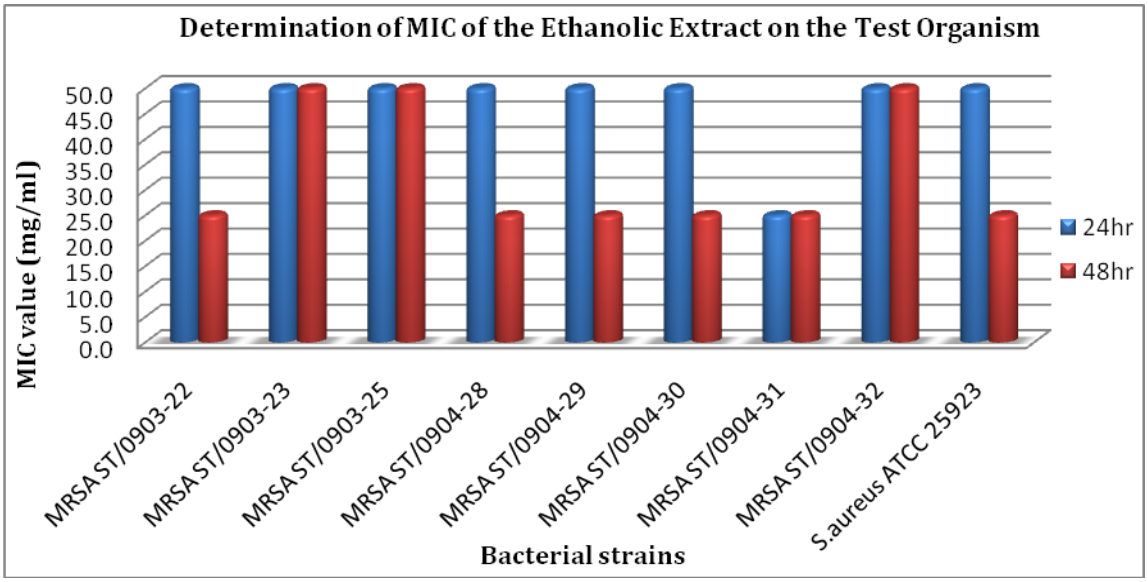


Figure 6: Effect of incubation period (24 and 48 hours) on *Tinospora crispa* ethanol extract by determination of MIC value. All –ST/09 strains denote MRSA strains

3.3 Antibacterial effects of several plant solvents

Several plant solvents were examined on tested organisms to ensure the absence of antibacterial effects by using set-up procedures similar to that of the MIC assay, at cell counts of approximately 5 x 10^5 CFU/ml. As a result, sterile distilled water and 10% (v/v) Tween 20 did not inhibit the growth of bacteria after 24 and 48 hours of incubation. However, the growth of the tested organisms was completely inhibited in all tubes by 99.9 % DMSO and by absolute ethanol as well.

3.4 Acute toxicity study

3.4.1 Mortality and behavioural changes

Within four hours after administration of the water and ethanolic extracts, no mortality occurred in all group. However, mortality occurred in all rats of 4 g/kg group after 72 hours administration of the aqueous extract and one male in 2 g/kg group after 96 hours administration of the aqueous extract **(Table 2)**. In addition, no mortality occurred in all other rats until the scheduled termination. No behavioural evidence of sign of toxicity was observed throughout the study period in all groups.

Table 2: Occurrence of mortality in acute toxicity study of *Tinospora crispa* extract

Dose	Extract type of *Tinospora crispa*	
	Aqueous	Ethanol
4 g/kg	**6/6***	0/6
2 g/kg	**1/6***	0/6
Vehicle	0/6	0/6

*Number of animals showing mortality after 72 and 96 hours of extract administration

3.4.2 Analysis of body weight

The changes of body weight of animals after the 14 days were recorded. **Figure 7 and 8** illustrated that there was no significant observed in any of rats groups when compared to their respective vehicle groups.

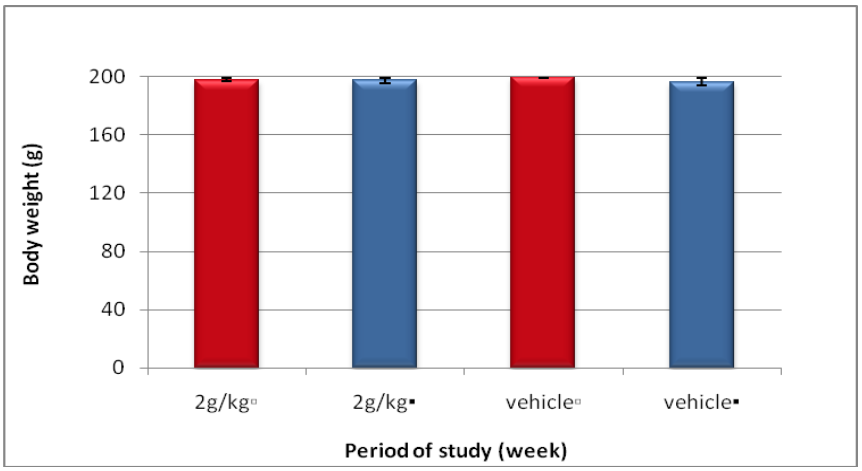


Figure 7: Mean body weights of rats tested with aqueous extract of *Tinospora crispa.*

▫Body weight at first day of experiment.

▪Body weight on 15th day of experiment.

No significant weight changes were observed in all groups.

Values are expressed as mean ± S.E.M.

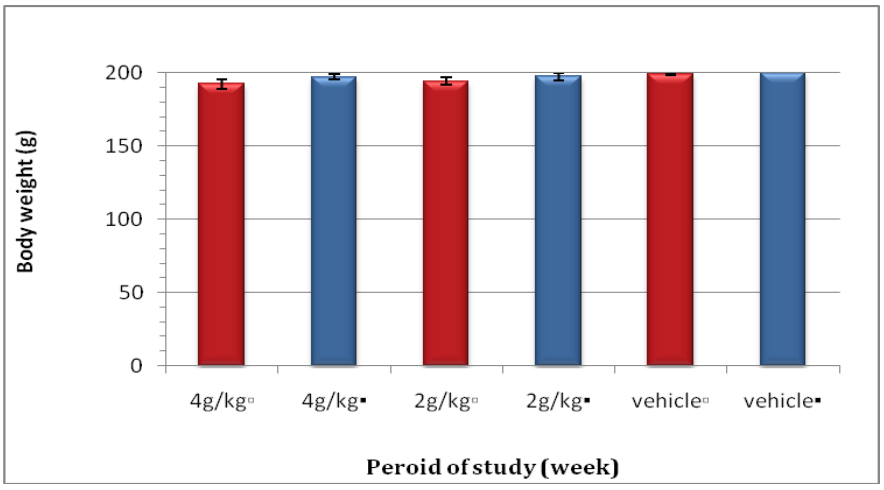


Figure 8: Mean body weights of rats tested with ethanol extract of *Tinospora crispa.*

▫Body weight at first day of experiment.

▪Body weight on 15th day of experiment.

No significant weight changes were observed in all groups.

Values are expressed as mean ± S.E.M.

3.4.3 Liver and renal function analysis

The parameter of liver function that had been tested was serum total protein, albumin, globulin, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatise. The levels of each of these enzymes were analysed for all groups and thereafter indications of liver function were compared to their vehicle groups. Creatinine and urea levels of all groups were determined as markers of kidney function.

In rat groups given the aqueous extract of *Tinospora crispa*, no significant increases were observed in levels of all tested parameters in all groups (**Figures 9** to **11**). Likewise, Levels of all tested parameters were not significantly increased between males or females groups as compared to the control groups. However, all rat groups given extract at high dose of 4g/kg died within 72 hours after administration.

Also, in rat groups given the ethanol extracts instead, no significant changes were found in all tested parameters levels of all rat groups as compared to their respective vehicle groups (**Figures 12** to **14**). Moreover, there was no a significant decrease or increase in levels of all parameters of male and as well as female groups in both doses.

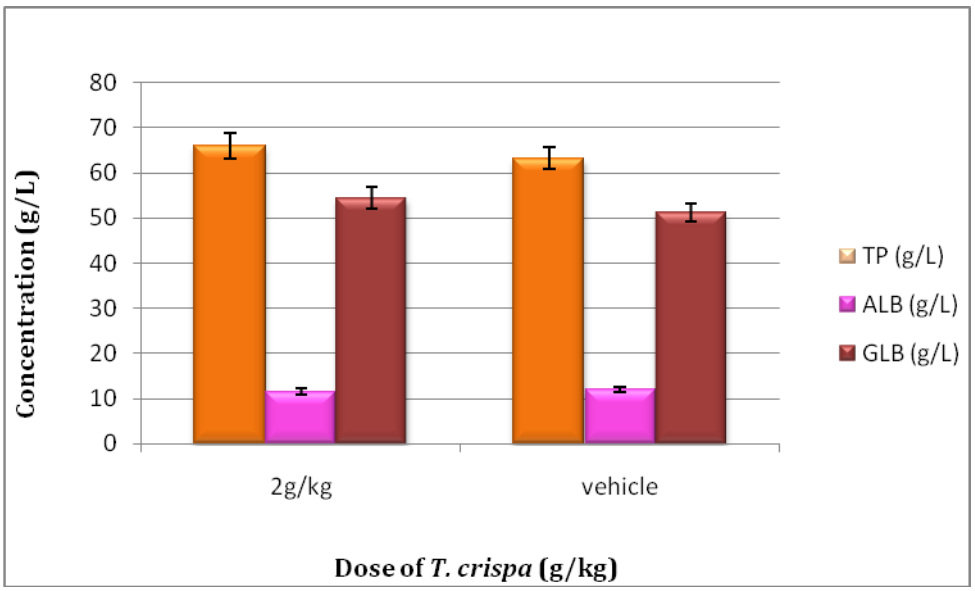


Figure 9: Total protein (TP), albumin (ALB), and globulin (GLB) levels in rats treated with aqueous extract of *T. crispa*

Values are expressed as mean ± S.E.M.

No significant difference as compared to vehicle group ($p<0.05$)

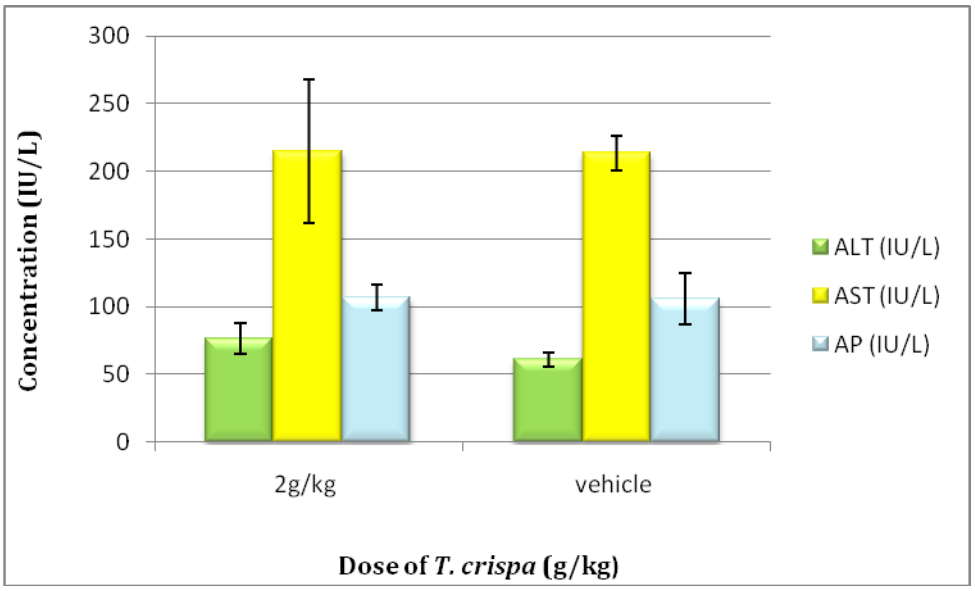


Figure 10: Levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (AP) in rats treated with aqueous extract of *T. crispa*

Values are expressed as mean ± S.E.M.

No significant difference as compared to vehicle group ($p<0.05$)

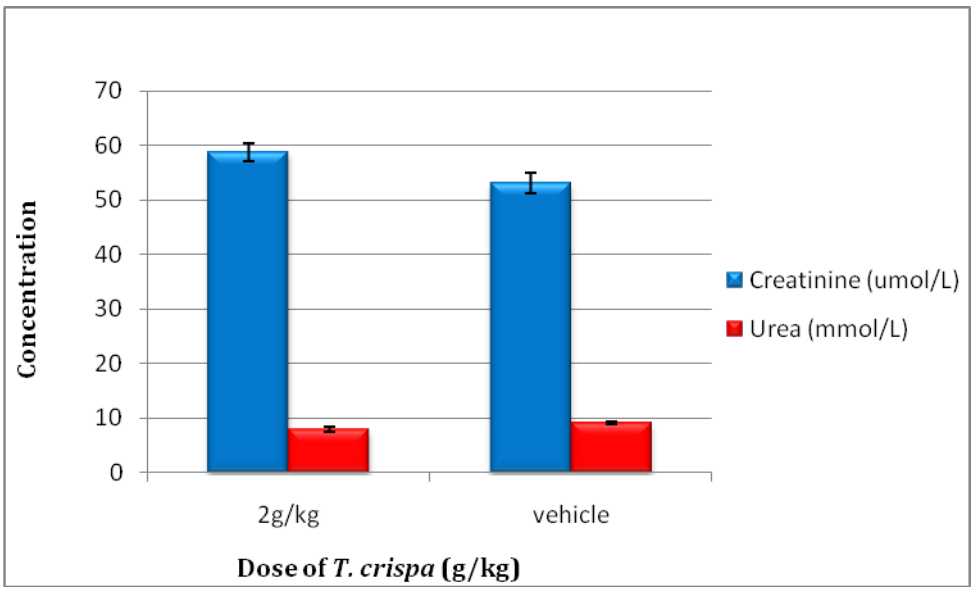


Figure 11: Creatinine and urea levels in rats treated with aqueous extract of *T. crispa*

Values are expressed as mean ± S.E.M.

No significant difference as compared to vehicle group ($p<0.05$)

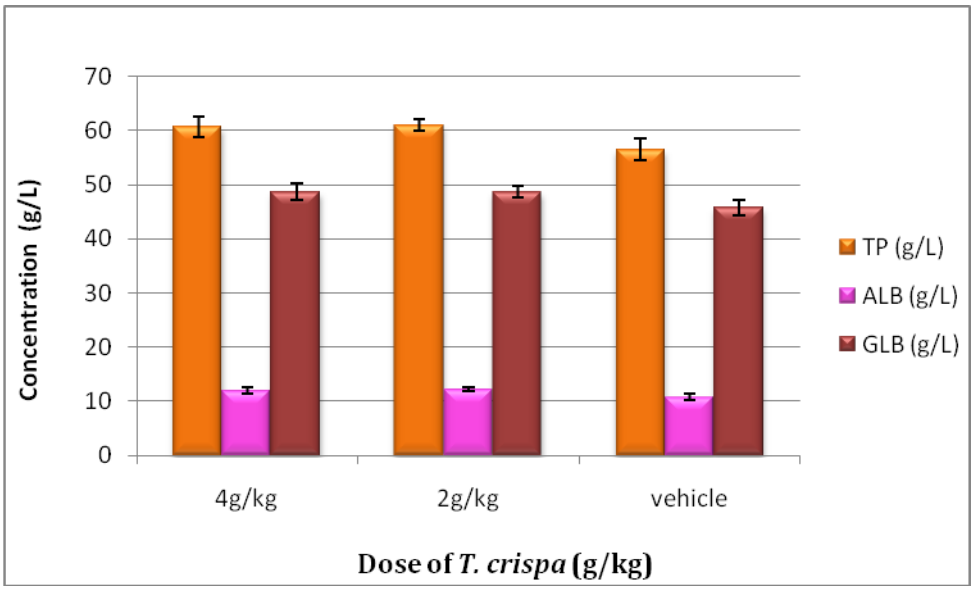


Figure 12: Total protein (TP), albumin (ALB), and globulin (GLB) levels in rats treated with ethanolic extract of *T. crispa*

Values are expressed as mean ± S.E.M.

No significant difference as compared to vehicle group ($p<0.05$)

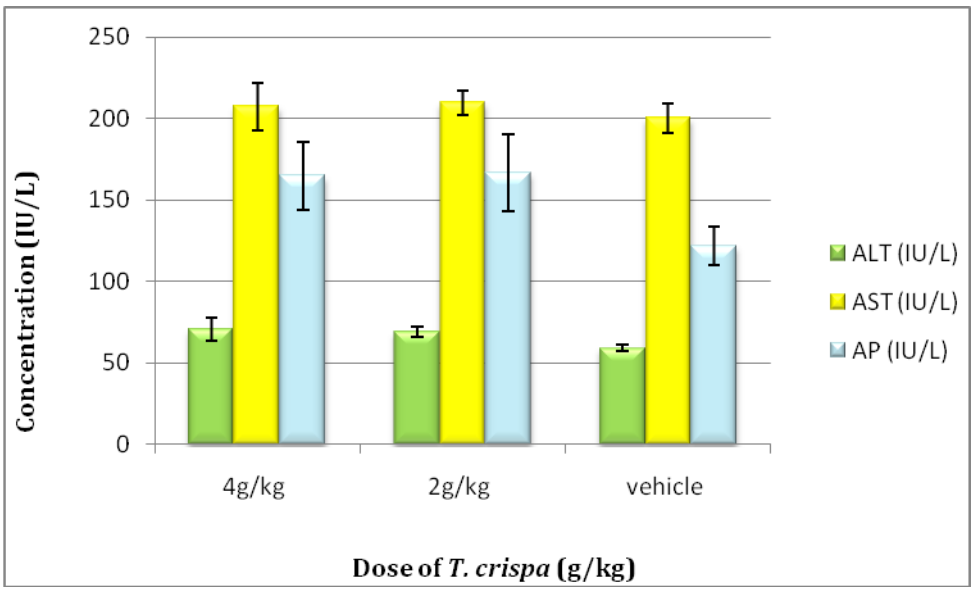


Figure 13: Levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (AP) in rats treated with aqueous extract of *T. crispa*
Values are expressed as mean ± S.E.M.
No significant difference as compared to vehicle group ($p<0.05$)

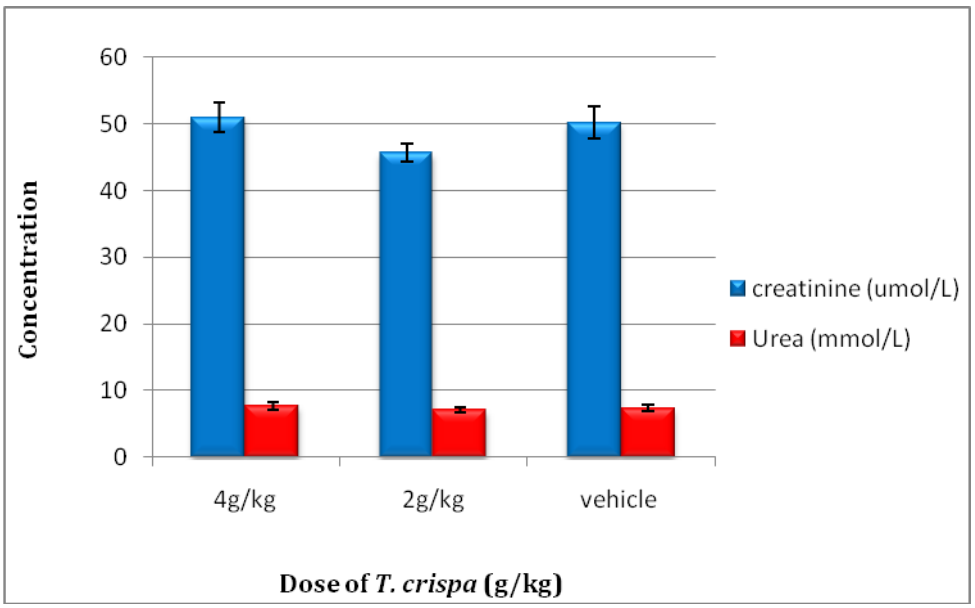


Figure 14: Creatinine and urea levels in rats treated with ethanolic extract of *T. crispa*
Values are expressed as mean ± S.E.M.
No significant difference as compared to vehicle group ($p<0.05$)

3.4.4 Gross necropsy and histology

Gross necropsy was performed before the livers and kidneys were excised. No gross pathological changes were observed in the organs of all animals. Generally, in histological examination, livers and kidneys of most dosage group animals exhibited normal architecture with the absence of pathological lesions. Liver lobules showed uninucleated and binucleated hepatocytes that radiate from the central vein to the periphery (**Figure 15 to 17**). Kidneys showed normal, distinct glomeruli and renal tubules (**Figure 18 to 20**). On the other hand, some of examined organs showed slightly changes in their architecture but were not significant when compared to their vehicle groups.

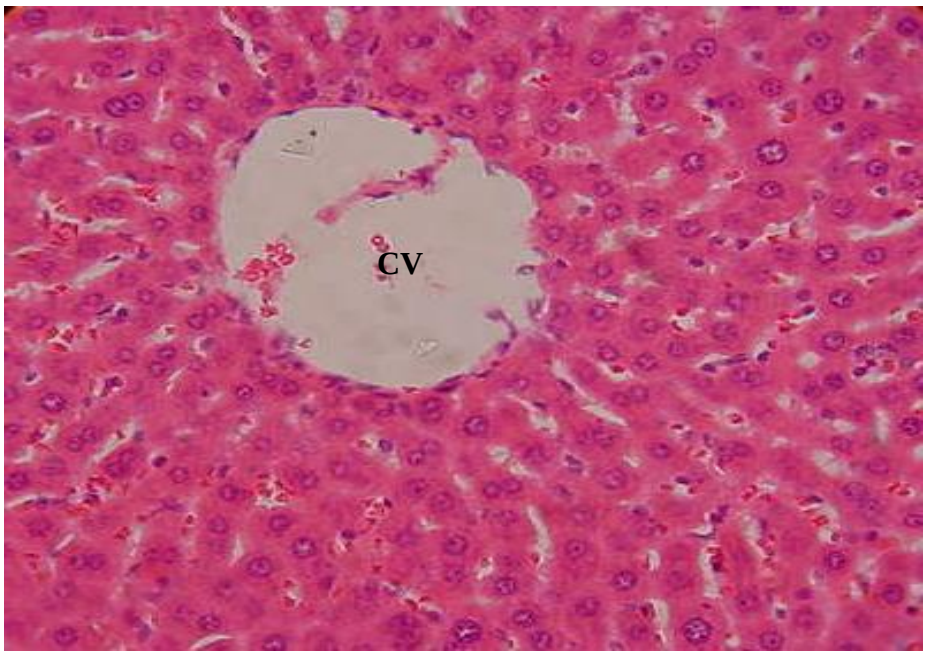


Figure 15: Histological section of liver parenchyma in rats treated with 10% (v/v) Tween 20 (vehicle control) showed normal architecture and no toxic effect to hepatic cells (H & E stain 40x). Microphotograph shows: central vein (CV).

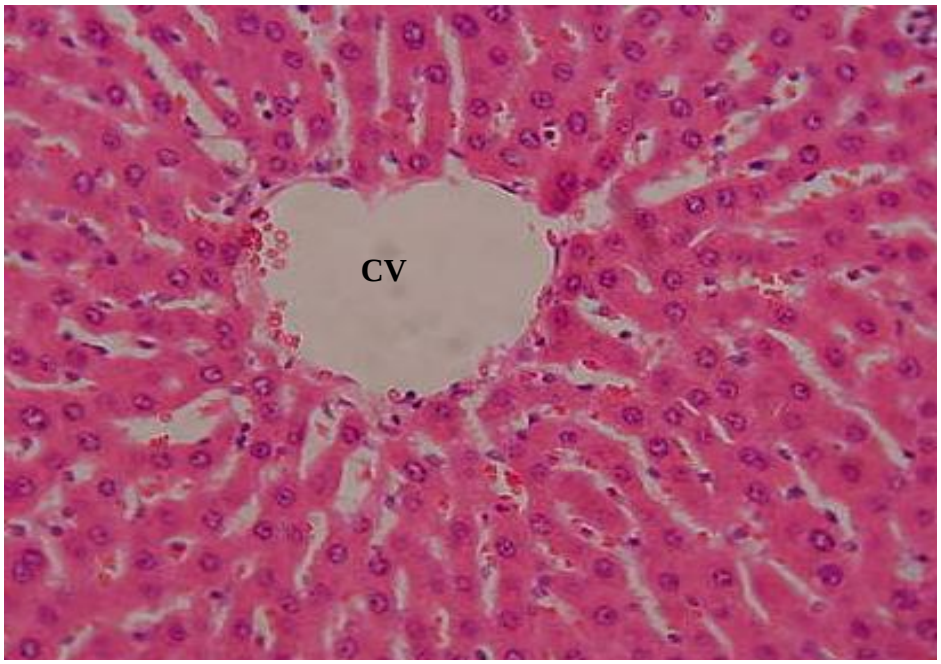


Figure 16: Histological section of liver parenchyma in rats treated with low dose aqueous extract of *T. crispa* (2 g/kg) showed normal architecture and no toxic effects to hepatic cells (H & E stain 40x). Microphotograph shows: central vein (CV).

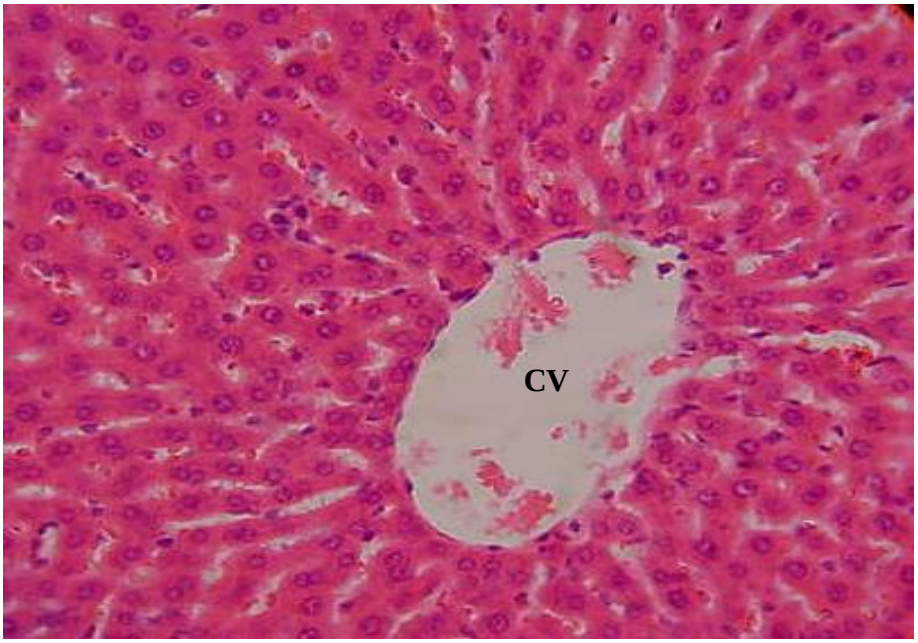


Figure 17: Histological section of liver parenchyma in rats treated with high dose ethanol extract of *T. crispa* (4 g/kg) showed normal architecture and no toxic effects to hepatic cells (H & E stain 40x). Microphotograph shows: central vein (CV).

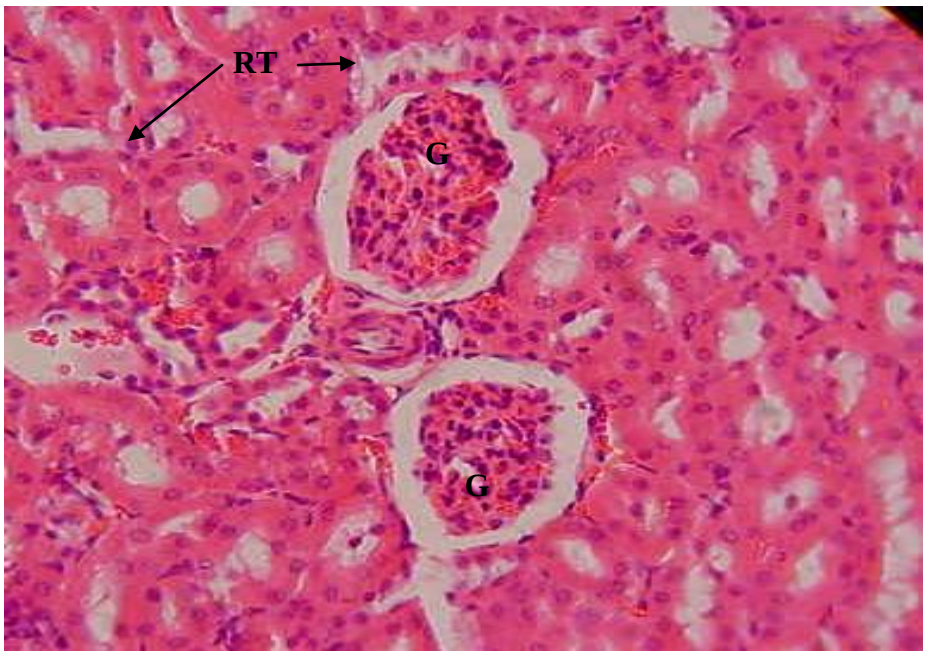


Figure 18: Histological section of kidney in rats treated with 10% (v/v) Tween 20 (vehicle control) showed normal tissue structure and no toxic effect to kidney cells (H & E stain 40x). Microphotograph shows: renal tubules (RT) and glomeruli (G).

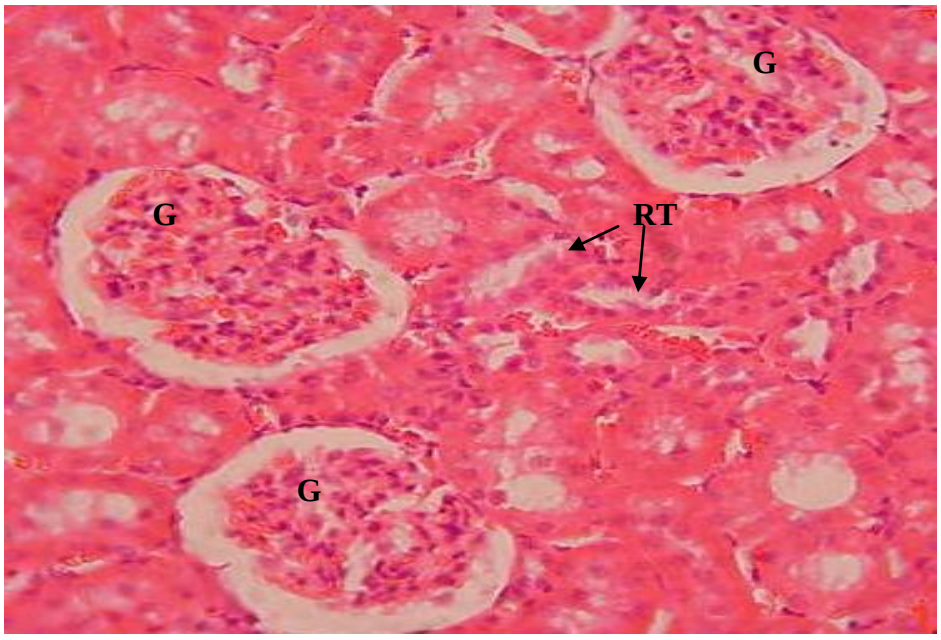


Figure 19: Histological section of kidney in rats treated with low dose of aqueous extract of *T. crispa* (2 g/kg) showed normal tissue structure and no toxic effect to kidney cells (H & E stain 40x). Microphotograph shows: renal tubules (RT) and glomeruli (G).

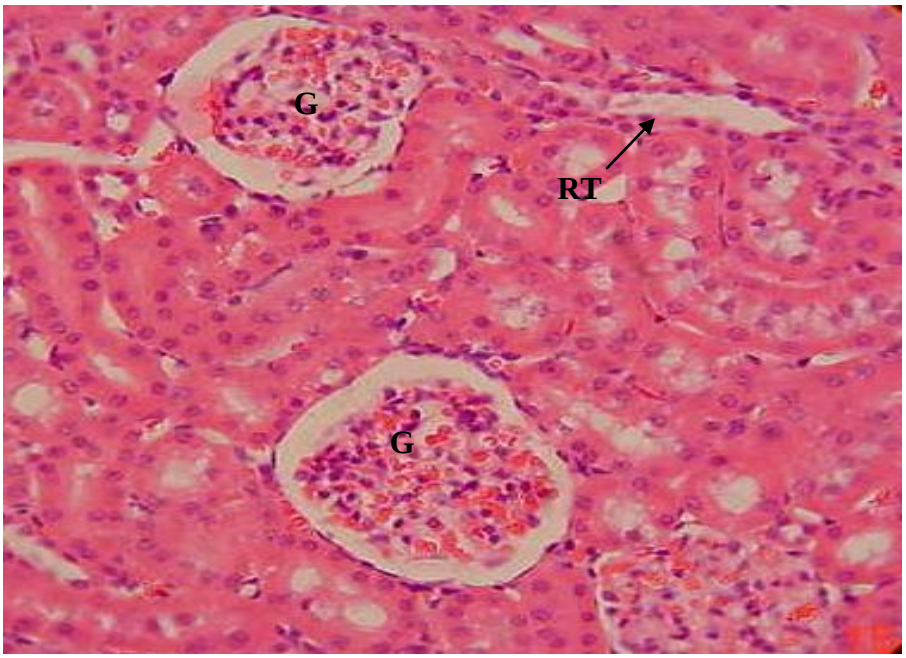


Figure 20: Histological section of kidney in rats treated with high dose of ethanol extract of *T. crispa* (4 g/kg) showed normal tissue structure and no toxic effect to kidney cells (H & E stain 40x). Microphotograph shows: renal tubules (RT) and glomeruli (G).

4.0 DISCUSSION

4.1 Experimental findings

4.1.1 Antimicrobial Susceptibility Test Method

Hospitals have shown a rising incidence of the resistance of *S. aureus* to methicillin and to a wide range of antibacterial agents which include all kinds of β-lactamase. This has made treatment designed to cure serious diseases more challenging (Abu-Shanab et al., 2006; Bhavnani & Tillostson, 2008). Vancomycin is the main therapeutic agent available to treat MRSA infectious, however there are reported cases showing some species of enterococcus and some coagulase negative staphylococci exhibiting resistance against vancomycin (Fraise, 1998; Hiramatsu, 2001; Assadullah et al., 2003). The recognition of vancomycin-intermediate *S. aureus* in Japan and the USA has lead to the recommendation of numerous approaches which are centered on natural compounds to be used in managing the outbreaks. (Fraise, 1998). The investigation of natural compounds as an alternative treatment has been used as a novel way to cure infectious diseases caused by MRSA (Abu-Shanab et al., 2006).

In the present study, the analysis of the growth inhibition activity by the disk diffusion method showed that the water extracts failed to inhibit growth of the test organisms at concentration 100 mg/ml, whereas the ethanolic extract of *T. crispa* possessed antibacterial activity and anti- MRSA against *S. aureus* ATCC and all the pure isolates of MRSA at the same concentration. This finding is in accordance with the reporting of Zakaria et al (2006), which found that the ethanolic extract of *Tinospora crispa* was bacteriostatic against *S. aureus* when used at a different concentration, while the water extract was not.

Determination of MIC allows for more accurate quantitative results of the antimicrobial strength of *T. crispa* as compared to the disc diffusion assay. It also allows more accurate comparison between aqueous and ethanol extracts. From the result of the broth dilution tubes for MIC, the aqueous extract of *T. crispa* was not inhibitory on all the tested organisms. On the other hand, alcoholic extract of this plant exerted inhibitory effect on the test organisms to different extents **(Table 1).**

It was observed that ethanol extract of the plant has potential antibacterial properties while water extract lacks of such property at the same concentrations. Researchers in the past had also revealed the same observation that ethanolic extract were more effective than water extract. According to many reported studies that ethanol is a better solvent when compared to water. This because ethanol has the stronger extraction capacity which it could have produced important number of antibacterial substances like tannins, saponins and alkaloids. Therefore, this study followed similar tendency (Akinyemi et al., 2005; Abu-Shanab et al., 2006; Sule & Agbabiake, 2008). In addition to the high volatility of ethanol, it also may be attributed to the nature of biological active compounds, i.e. tannins, and alkaloids, well known for antimicrobial activity, which could be produced if ethanol was used as a solvent (Akinyemi et al., 2005).

According to Sudjana et al., 2009, the tested organisms were inhibited by ethanol extract, thus this indicated that the plant extract acted specifically against the gram positive cell wall due to its effectiveness against all the staphylococcal strains. The reason may be due to their hydrophobicity that causes cell eruption as a result of the destruction of the structure of the membrane (Nitta et al., 2002). When the aqueous extract was used, there was an absence of positive result but this does not necessarily imply an absolute absence of bioactive compounds, as there may be other compounds in the extract that are exerting opposing effects against these bioactive compounds (Eldeen

et al., 2005). In addition to this, it may be due to the lack of alkaloids which have been associated with antibacterial activity of the aqueous extract of *T. crispa* steam (Dweck & Cavin, 2006). Even though in some cases, the bacteria itself may produce capsules that are less soluble in the water (Sule & Agbabiake, 2008).

From disc diffusion test, we observed that the diameters of inhibition zone did not surpass that of the vancomycin positive controls. This may be due to the effects of agar medium on the diffusion of the active compounds, insufficient concentration of the antibacterial compound, or it could have been due to the extract impurity as only crude extracts were used in the experiment. Therefore, purification of the potential antibacterial agent may increase its relative activity, i.e. give bigger zones of inhibition than the positive controls or lower MIC values than those obtained from this study (Pesewu et al., 2008). However, the identification of the responsible compound is beyond the scope of this project.

The result of this investigation was different when compared to findings from other studies that had used the same plant for its antibacterial property screening. There are several factors which could have influenced the result namely: different extraction methods used, the different strains of tested bacteria (Pesewu et al., 2008), and plant materials sources and the part of the plant used for extraction. In this study, the whole plants were used. According to Yu et al (2007), the different parts of a plant may contain different chemical components that contribute to the strength of its antimicrobial activity.

Moreover, there are several factors that could affect the results of the disc diffusion testing. First of all, the diameter of the inhibition zones are affected by the rate of diffusion of the antimicrobial compound and thus may not accurately represent the strength of the extract's antimicrobial activity. Also, another factor is the size inoculums standardization to achieve 0.5 McFarland turbidity. Indeed, inoculum size is very

important to ensure uniform lawn growth, as a smaller inoculum size may produce falsely large inhibition zones while a bigger inoculum size may produce falsely smaller zones instead (Jorgensen & Turnidge, 2007).

The solvents were chosen based on degree of solubility of the ethanol extract, and also tested to determine whether they possess antibacterial effects or not. Absolute ethanol and DMSO being the most effective solvents followed by 10% (v/v) Tween-20, and finally distilled water. DMSO and absolute ethanol completely inhibited bacterial growth. If these two solvents were chosen for the MIC assay, the results obtained would have been absolutely false. Therefore, 10% (v/v) Tween-20 was the solvent chosen to dissolve the ethanolic extract of plant in. Solubility of the extract in the chosen solvent is important to ensure that the correct concentration of the extract solution was prepared.

One potential problem with using *T, crispa* extracts as an antibacterial agent is the cytotoxicity. It has been found that the viability of normal cell lines decreased gradually when *T.crispa* used as therapy (Zulkhairi et al., 2008). Similar result was also obtained from the study by Chantong and co-workers (2008), those have found ethanolic extract showing high cytotoxic effect when was tested on mouse P19 embryonal carcinoma cells using XTT.

4.1.2 Acute toxicity study

Up to 80% of the population in developing countries has widely used herbal medicine (Hilaly et al., 2004), such as *Tinospora* species are extensively used in Asia and Africa as a medicinal plant (Kongkathip et al., 2002). Thai people use this plant intensively for a long time as a bitter tonic, antipyretic (Chavalittumrong et al., 1997), and as well as a cure for diabetes (Pannangpetch et al., 2006). Despite the widespread use, few scientific studies have reported the security and effectiveness of the plants as therapeutic agents (Hilaly et al., 2004). Hence, a cute oral toxicity study (OECD 420)

was carried out to examine the potential toxic effects, and evaluate the safety of this extract for human consumption, and for pharmaceutical preparation to be used in humans (Pannangpetch et al., 2006).

The 14-day observation period was recommended by the OECD guidelines for observation of any incidences of delayed toxicity or death, or to observe the existence and the disappearance of toxic effects. In this study, at the highest dosage of 4g/kg/ BW, mortality was observed in one group where all the rats died within 72 hours after a single oral administration of aqueous extract of *T. crispa*. Likewise, the death of one rat was recorded after 96 hours in group received aqueous extract of *T. crispa* at the dose of 2g/kg/BW. However, other animals in the same group did not exhibit any mortality or moribund statuses, this defined according to OECD, (2001) as "being in a state of dying or inability to survive even if treated". In contrast to the first group, only one rat died from the second group. According to our observations this could not have been due to the toxicity in the extract but other reasons such as the rat being ill.

However, past studies discovered that the water extract of *T. crispa* stem induce basal potentiation and the secretion of glucose stimulated insulin from rat and from islets of Langerhans human as well. In addition, the aqueous extract of its steam at doses of 100 to 300 mg/kg has been found to reduce the fever in rats (Chavalittumrong et al., 1997). The hypoglycaemic effect of the aqueous extract of *T. crispa* in moderately diabetic rats was observed with containment enhancement in insulinemia at dose of 4 g/l in the drinking water and the levels of plasma insulin has been observed to increase when extract at dose of 50 mg/kg was given with acute intravenous treatment (Noor & Ashcroft, 1989). From various studies, we have investigated that the aqueous extract of *T. crispa* steams has been used in small amount which it could be suggested that aqueous extract is fairly non-toxic at small dose of the extract and/ or it could be due to the parts of a plant that have been used. In this study, the whole plant was used and

some parts consist of different chemical components (Yu et al., 2007), so some of the compounds of certain parts could be responsible for the toxicity effects and mortality in group given aqueous extract at high dose.

After administering a single oral dose of absolute ethanol extract at high and low dosage of 4, and 2g/kg/BW respectively, none of the rats exhibited any behavioral sign of toxicity or mortality however this was not the case with those from the aqueous to ethnolic extract. This gives an indication that the exposure to absolute ethanol extract does not result in acute oral toxicity. This finding is in agreement of the finding by Chavalittumrong et al. (1997) ,where all the mice that were given ethanolic extract at doses of 1, 2, and 4g /kg/ BW did not produce any signs of toxicity or mortality. Thus, it was observed that ethanolic extract of *T. crispa* was safe at a dosage of up to 4g/kg/ BW. It is important to note that even though mortality and insignificant behavioural changes were not observed, it may not necessarily be safe. This can be as a result of the extracts being poorly absorbed from the gastrointestinal tract or quickly metabolized to less toxic metabolites (Hilaly et al., 2004).

The effects on body weight and food consumption in relation to the extract only show a slight change in average body weight in all groups and as such were not recorded due their insignificance when compared to their respective control group. Therefore this gave an indication that the extracts had no effect on food intake or weight loss in the animals. However, all rats in the group receiving the aqueous extract at a dosage of 4g/kg/ BW which died after 72 hours of administration had less appetite and as a result were less active.

The analysis of the parameters for liver function such as serum total protein, albumin, globulin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase were evaluated. From the given parameters, ALT, and AST levels were the two which were considered as good marker enzymes for liver function

(Hilaly et al., 2004; Mehta et al., 2009). In the event that high levels of hepatospecific enzymes such as AST and ALT were released in serum, it would be indicative of damage to the hepatic cells of the liver (Kumar et al., 2009). A decrease in the amounts of total protein, albumin and globulin is indicative of chronic liver damage since the majority of plasma proteins such as albumin and different globulins are synthesized in the liver. Thus this may be used to evaluate the synthesizing capacity of the liver (Rasekh et al., 2008; Mehta et al., 2009). A severe histological change in the liver can be pointed out by a raise in the level of serum alkaline phosphatase and secretion of large amounts into plasma (Sharma et al., 2008).

To evaluate the renal function, urea and creatinine were the parameters analyzed (Hilaly et al., 2004). The two are usually considered as marker substances of kidney function (Feres et al., 2006). Thus significant increases in levels of renal enzymes are conventional indicators of kidney damage or nephrotoxicity (Sharma et al., 2008). In the current study, from the observation of the effects of both extracts of blood chemistry, there were insignificant differences in the liver and renal function parameters in all treated groups, and between genders as well, when compared to their respective control group. The lack of difference in reaction to the toxicity between the control and treated groups could be attributed to the period of exposure to the extract which is only two weeks (according to OECD guideline).

The effects of both extracts on histopathology of internal organs were investigated using the livers and kidneys. The two organs were chosen because the oral route of administration was used and the presence of any sign of toxicity would be most obvious in those organs. The liver is generally referred to as the detoxification organ because it is made up of endoplasmic reticulum of the hepatocytes which is responsible for the degradation of toxic substances. This means that when a toxic substance is administered, the hepatic cell would wind up with lethal damage. The kidney is an

organ (Rasekh et al., 2008) of the glomerulus site, which is the most sensitive structure, as it is the main site for several chemical actions it thus would be damaged by toxic substances (Sharma et al., 2008). Generally, according to the current study upon examination of the organs did not reveal the presence of pathological lesions in all the groups, although there were some modifications in the organs architecture which were not as significant when compared to their respective control groups. Hence a single dose of the extract indicates the absence of cytotoxic effects but at repeated doses there is a high chance that cytotoxic effects may occur.

4.2 Limitation of the study

The limitations faced in this study need to be addressed and further studies suggested for this plant. For the antimicrobial testing, time did not permit screening of other bacterial strains. There was sufficient extracts for only determination of MIC as a result, determination of the minimum bactericidal concentration (MBC) was unable to be carried out, which when coupled with the MIC, would have helped indicate whether the antibacterial effect of the extract was bacteriostatic or bacteriocidal (Okusa *et al*, 2007; Yu et al., 2007). Extraction type and part of the plant used for extraction could not be thoroughly investigated in this study due to time and budget constraints.

For the toxicity studies, the experiment was done according to the OECD guidelines. However given more time in-depth findings using repeated dose, sub-chronic and chronic studies may be carried out to further justify the use of *T. crispa* as a safe herbal medicine.

4.3 Future study

Susceptibility testing of the antimicrobial properties of *T. crispa* on other organisms such as fungi and parasites should be carried out. Further investigation also

should be carried out to isolate and identify the responsible compounds that cause inhibitory effects of the ethanol extract and toxicity effects which cause mortality, of aqueous extract. Other parameter such as repeated dose, sub-chronic and chronic studies need to be carried out and are thus suggested for further studies to investigate for long-term, accumulative effects of *T. crispa* administration.

5.0 CONCLUSION

The rises in antimicrobial resistance possess a growing concern globally. Hence the discovery of novel antimicrobial drugs from medicinal plant as alternative valuable therapeutic agents is important. The result presented in this study revealed that the ethanolic extract of *T. crispa* has antibacterial effect on all the tests organisms whereas water extract failed to inhibit the growth of all the test organisms at the same used concentration. From acute oral toxicity study, ethanolic extract of *T. crispa* appears to have low toxic effects with an LD_{50} value that exceeds 4g/kg, while the LD_{50} of aqueous extract have to be less than the administered dose of 4g/kg. Indeed, more studies need to be done to identify the responsible antimicrobial compounds as well as to test for toxicity after prolonged consumption.

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7.0 APPENDICES

APPENDIX A: Gram staining protocol

1. Smear the bacteria on glass slide.
2. Flood the slide with crystal violet solution for 1 minute, and then drain solution off with distilled water.
3. Flood slide with Lugol's iodine for 1 minute, and then drain solution off.
4. Rinse slide with acetone (decolourizer) for several seconds.
5. Drain solution thoroughly with distilled water until the excess colour goes off.
6. Flood slide with safranine for 1-2 minutes, and then wash off the stain with distilled water.
7. Leave slide to air-dry.
8. Observe slide microscopically under oil immersion.

APPENDIX B: Histology protocol

1. Specimen preparation
2. Automated Tissue Processing
3. Tissue blocking
4. Sectioning
5. Staining
6. Mounting

1. Specimen preparation

- Label biopsy tissue properly
- Fix organs (kidney and liver) by placing in 10% neutral buffered formalin solution immediately after excision for approximately 6 hours.
- Next, trim the tissues into small size and place in labelled cassettes.
- Then, place the cassettes in the 10% neutral buffered formalin again for further fixation overnight before processing.

2. Automated tissue processing (Leica TP1020)

Process	Solution	Period (hour)
Fixation	10% formalin I	1
	10% formalin II	1
Dehydration	70% ethanol	1
	95% ethanol I	1
	95% ethanol II	1
	95% ethanol III	1
	Absolute ethanol I	1
	Absolute ethanol II	1 ½
Cleaning	Alcohol : xylene (1:1)	1
	Xylene I	1 ½
	Xylene II	1 ½
Infiltration	Paraffin wax I	1 ½
	Paraffin wax II	1 ½

3. Tissue embedding (Leica HISTOEMBEDDER)

- Place and orientate the tissue in mould.
- Pour paraffin wax into mould.
- Place mould in cold plate area until paraffin wax solidifies
- Pry tissue block off of mould for sectioning.

4. Sectioning (Leica RM 2135)

- Section the tissue blocking by using a rotary microtome at a thickness of 5μm
- Place ribbon sections in water bath.
- Pick two best sections from water bath with separate, clean slides.
- Adherence the sections by placing glass slide on hot plate.

5. Staining

a. Bring section to water:

Process	Solution	Period (minutes)
Dewaxing	Xylene I	3
	Xylene II	3
Rehydration	Absolute alcohol	2
	95% alcohol I	2
	95% alconol II	2
	70% alconol	2
Bring section to water	Running tap water	3

b. Staining with Haematoxylin & Eosin:

Process	Solution	Period
Staining	Haematoxylin stain	10 minutes
	Running tap water	until excess colour goes off
Differentiation	0.5% acid alcohol	2-3 dips
	Running tap water	2-3 minutes
	2% sodium acetate	2 seconds
	Running tap water	2-3 minutes
	80% alcohol	2-3 dips
Staining	Eosin stain	5 minutes
Dehydration	95% alcohol I	5 seconds
	95% alcohol II	2 minutes
	Absolute ethanol I	2 minutes
	Absolute ethanol II	2 minutes
Cleaning	Xylene I	2 minutes
	Xylene II	2 minutes
	Xylene III	2 minutes

6. Mounting with DPX

- Mount slides with DPX mounting media and cover the section with cover slip.
- Wipe slide to remove excess xylene and then observed under light microscope**.**

APPENDIX C: Data tables

Table (i) Mean diameters of zone in agar plate against nine bacteria strains by ethanolic extract of *Tinospora crispa*

Bacteria strain	Mean diameters (mm)[a] of inhibition zones in			
	All used 10, 30, and 50 µl of 100 mg/ml			
	10µl	30µl	50µl	Vancomycin (30µl) [b]
MRSA ST/0903-22	10.7	11.3	12	19.3
MRSA ST/0903-23	11.3	12.0	12.3	19.3
MRSA ST/0903-25	12.7	13.3	13.3	20
MRSA ST/0904-28	11.3	11.7	12	17.6
MRSA ST/0904-29	12.0	12.7	12.7	18
MRSA ST/0904-30	13.0	13.3	13.5	19.6
MRSA ST/0904-31	13.0	13.3	13.3	20
MRSA ST/0904-32	12.0	12.7	13	19.3
S.aureus ATCC 25923	7.7	10.3	11.3	18.3

(-) Indicate absence of any observable inhibition zone.

[a] Values of each represent the mean of three replicates.

[b] Zone diameters of positive controls.

DMSO was used as a negative control.

Table (ii) Body weights of rats in acute toxicity of aqueous extract of *Tinospora crispa*

Dose	Body weight (g) day 0	Body weight (g) day 15
4g/kg	198.00 ± 1.23	-
2g/kg	197.80 ± 1.01	197.40 ± 1.66 [a]
Vehicle	199.50 ± 0.50	196.50 ± 2.36

Values expressed as mean ± S.E.M., n = 6, [a] n = 5

No significant body weight changes observed in all rats groups

(-) No value due to the death of rats of this group within 72 hours

Table (iii) Body weights of rats in acute toxicity of ethanolic extract of *Tinospora crispa*

Dose	Body weight (g) day 0	Body weight (g) day 15
4g/kg	192.33 ± 3.06	197.16 ± 1.66
2g/kg	194.33 ± 2.59	197.33 ± 2.66
Vehicle	199 ± 0.70	199.75 ± 0.25

Values expressed as mean ± S.E.M., n = 6

No significant body weight changes observed in all rats groups

Table (iv) Liver function analysis of rats in acute toxicity study of aqueous extract of *Tinospora crispa*

Dose	TP (g/L)	ALB (g/L)	GLB (g/L)	ALT (IU/L)	AST (IU/L)	AP (IU/L)
4g/kg	-	-	-	-	-	-
2g/kg [a]	66.00 ± 2. 82	11.60 ± 0.67	54.40 ± 2.37	76.60 ± 11.66	215.00 ± 53.11	106.80 ± 9.51
Vehicle	63.28 ± 2.49	12.00 ± 0.57	51.25 ± 2.09	60.75 ± 5.17	213.75 ± 12.74	106.00 ± 19.10

Values expressed as mean ± S.E.M., n = 6, [a] n = 5

Parameters tested: TP: total protein; ALB: albumin; GLB: globulin; ALT: alanine aminotransferase; AST: aspartate aminotransferase and AP: alkaline phosphatise

(-) No value due to the death of rats of this group within 72 hours

Table (v) Liver function analysis of rats in acute toxicity study of ethanolic extract of *Tinospora crispa*

Dose	TP (g/L)	ALP (g/L)	GLB (g/L)	ALT (IU/L)	AST (IU/L)	AP (IU/L)
4g/kg	60.67 ± 1.80	12.00 ± 0.51	48.66 ± 1.47	70.50 ± 6.86	207.50 ± 14.63	164.66 ± 21.11
2g/kg	61.00 ± 1.12	12.33 ± 0.81	48.66 ± 0.98	69.16 ± 3.23	209.66 ± 7.25	166.50 ± 23.53
Vehicle	56.50 ± 1.93	10.75 ± 0.62	45.75 ± 1.43	59.00 ± 2.08	200.25 ± 9.25	121.75 ± 11.77

Values expressed as mean ± S.E.M., n = 6

Parameters tested: TP: total protein; ALB: albumin; GLB: globulin; ALT: alanine aminotransferase; AST: aspartate aminotransferase and AP: alkaline phosphatise

Table (vi) Renal function analysis of rats in acute toxicity study of *Tinospora crispa* extracts

Dose	Creatinine (umol/L)	Urea (mmol/L)
4g/kg[b]	-	-
2g/kg[b]	71.60 ± 12.93[a]	7.90 ± 0.51[a]
Vehicle	53.00 ± 1.87	9.05 ± 0.22
4g/kg[c]	51.00 ± 2.16	7.70 ± 0.62
2g/kg[c]	45.66 ± 1.35	7.10 ± 0.40
Vehicle	50.25 ± 2.42	7.35 ± 0.54

Values expressed as mean ± S.E.M., n = 6, [a] n = 5

[b] Aqueous extract of *Tinospora crispa*

[c] Ethanolic extract of *Tinospora crispa*

No significant body weight changes observed in all rats groups

(-) No value due to the death of rats of this group within 72 hours