

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. crispata* stem (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 stem 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* stems 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 ethanolic 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. crispera* 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.