9.0. GENERAL DISCUSSION

Malaria is one of the most important health problems in tropical and sub tropical countries. The World Health Organization estimates that, 2300 million (41%) people of total world population have been living at high malaria risk. More than 300-500 million clinical cases have been reported annually resulting in at least 1.5-2.7 million deaths. Among them, one million children under 5 years old are affected only by malaria or in combination with other diseases (WHO, 1997, 1998). This is mainly due to non availability of effective medicine and development of newer Plasmodium strains. To overcome these problems, development of effective newer drugs without showing side effects is need of the hour. Identification of potential drugs from living organisms particularly from marine organisms could solve several hurdles in the management of malaria. Among the marine organisms, marine animals have been identified as a good source for drug development. Due to lack of cultivation technology and multiple years taken for the production of effective bioactive principles could not meet out the urgent demand of antimalarial drugs. Marine invertebrates have developed highly specific relationship with numerous associated microorganisms and these associations are of recognized ecological and biological importance (Armstrong et al., 2001; Strahl et al., 2002). It has been reported that, the ratio of microorganisms with antimalarial activity from invertebrates were higher
than other sources (Ivanova et al., 1998; Burgess et al., 1999), which suggest that invertebrate associated microorganisms might play a chemical defence role for their host. This kind of microorganisms as a sustainable resource has high potential to biosynthesis more biologically active secondary metabolites with in a short span of time due to well developed cultivation technology. Bioactive natural products from sponges have been reported to have strong bioactivities including anticancer, antimicrobial and anti-inflammatory activities and are often applicable for medical use (Faulkner, 2002; Pettit et al., 2004). Unlike other invertebrates, sponges harbour extraneous microorganisms on their surface in their canal system and in the intracellular matrix which constitute a large part of the body as much up to 40% of the total biomass (Santavy et al., 1990). Earlier investigations reveals that, many compounds found in sponges are biosynthesized through microorganisms associated with them or indeed produced by microorganisms (Bewley and Faulkner, 1998). To confirm this hypothesis, there has been a great deal of interest in isolating bioactive microorganisms from sponges (Mitova et al., 2003; Suzumura et al., 2003). With the aim of finding new bioactive compounds from marine microorganisms, the present study has initiated to isolate bacteria and actinomycetes showing potential antiplasmodial activity associated with variety of sponge species distributed along the coast of Palk Strait.
The present study has collected 36 sponge samples throughout the year at different seasons distinguished by colour and ornamentation. All the samples were critically subjected for the isolation of bacteria and actinomycetes by following standard procedures. It reveals that, all the sponge samples have reported to harbour either group of microorganisms. It is surprised to notice that, 22 sponge samples are not reported to harbour actinomycetes. This is because 0.1% of microbes from these sponge were amenable to culture using traditional techniques and therefore the vast majority of microorganisms associated with sponges particularly actinomycetes could not be identified using a culture based approach. The present study also observed that, the bacteria is found to be a dominant group of microorganisms associated with sponges than the actinomycetes.

**Relative composition of sponge associated actinomycetes and bacteria**

![Pie chart showing the relative composition of sponge associated actinomycetes and bacteria. Bacteria: 87.57%, Actinomycetes: 12.43%]
Burja and Hill (2001) reported that, 228 strains of bacteria, 25 fungi, 3 actinomycetes and 9 strains of cyanobacteria were isolated from 10 individuals of sponge samples from Australian Great Barrier Reef. This study supports the results of the present study that, the bacteria are the dominant group of microbes irrespective to the seasons and the collection sites. The present study also found that, the bacterial groups were found maximum during monsoon season (November-January). This might be due to the higher nutrient derived from the fresh water runoff from the adjacent river which supports the maximum growth of bacteria during rainy season. The rain fall data from the local meteorological station shows that 9.75 mm highest rainfall had been reported during the period of collection when compared with the other places in Palk Strait. In contrary, the actinomycetes counts were found maximum during the summer season (May-July). This might be due to the availability of huge amounts of dissolved and particulate nutrients introduced into the sea during monsoon by land runoff could deposit the nutrients during summer (Wilkinson and Garonne, 1980). Besides that, microbial sponge associates depends upon the transportation of waste products or active metabolites (Borowitzka et al., 1989), chemical defence (Unson et al., 1994) or contribution to mechanical structure (Wilkinson, 1978b).
The present study also made an attempt to screen the morphologically different bacteria and actinomycetes strains against the chloroquine sensitive *Plasmodium falciparum* strains. This reveals that, all the bacteria and actinomycetes strains showed suppression of parasitemia. Of the 76 strains of bacteria THB-14 showed maximum suppression of parasitemia at a concentration of 200 μg.ml⁻¹. This might be due to the presence of important biochemical constituents present in the crude extracts. It is observed that, the crude extracts obtained from Bacterium RJAUTHB-14 is reported to have alkaloids and tannins (Table 29).

Likewise, out of 20 actinomycetes strains ACT-20 showed maximum suppression of parasitemia. The inhibition of parasitemia might be due to the presence of unique biochemical constituents such as reducing sugar, phenol and alkaloids present in the crude extract of *Streptomyces sp.* RJAUACT-20 (Table 29). Stierle *et al.*, (1988) reported that, the presence of alkaloids, phenols and reducing sugars showed potential *in vitro* antiplasmodial activity. Alkaloids are one of the major physiologically-active nitrogenous compounds derived from many biogenetic precursors, possessing antimalarial activity (Saxena *et al.*, 2003). Number of alkaloids, manazamine (Sakai *et al.*, 1986; Peng *et al.*, 2008), neo-kauluamine (El Sayed *et al.*, 2001), homofascaplysin A (Kirsch *et al.*, 2000); phloecdiktynes (Kourany Lefoll *et al.*, 1992; Mancini *et al.*, 2004), 6-Bromoaplysinospsin (Hu *et al.*, 2002); heptyl
prodigiosin (Lazaro et al., 2002); salinosporamide A (Feling et al., 2003) from marine sources have been reported to possess antimalarial activity.

Moreover, alkaloid derivatives viz., 8-hydroxy-manzamine, manazamine (Russell et al., 2004), cycloprodigiosin (Kim et al., 1999), heptyl prodigiosin (Lazaro et al., 2002), ascosalipyrrrolidinone A (Osterhage et al., 2002) were reported from marine microbial community. Moreover, phenolic groups are highly hydroxylated which includes hydroxylcoumarins, hydroxycinnamate derivatives, flavanols, flavonols, flavanones, flavones, anthocyanins, proanthocyanidins, hydroxystilbenes, aurones, etc. Many of the polyphenols are well documented to treat chronic diseases such as cardiovascular disease, cancer, diabetes, bacterial and parasitic infections (Murakami et al., 1994; Sherman and Billing 1999; Scalbert, 1991; Cowan, 1999). The antiplasmodial activity of marine sponge associated microbial alkaloids (curcuphenol) has been reported by El Sayed et al. (2002). Otoguro et al. (2004) reported that, polysaccharides, polyketides and polysaccharide derivatives (Prumycin) are having potential antiplasmodial activity.

The present study also observed that, the suppression of parasitemia is increasing with the increasing concentration of active principles and also increasing the duration of incubation. It is also important to notice that, the actinomycetes are found to be more potent (IC$_{50}$ 3.125 μg.ml$^{-1}$) in the
suppression of parasitemia than the bacteria (IC\(_{50}\) 6.5 \(\mu\)g.ml\(^{-1}\)). Actinomycetes have proved to be having the rich source of secondary metabolites and the most of the commercial antibiotics have been isolated from actinomycetes (Sanglier et al., 1996). Several workers have isolated actinomycetes from the marine environment, some of the actinomycetes showed antibacterial activity (Fenical and Jensen, 1993), antitumor activity (Baz et al., 1997) and antiplasmodial activities (Webster et al., 2001). According to Gessler et al. (1994), if the extracts displayed an IC\(_{50}\) <10 \(\mu\)g.ml\(^{-1}\) is very good antiplasmodial activity, from 10 to 50 \(\mu\)g.ml\(^{-1}\) antiplasmodial activity was moderate and over 50 \(\mu\)g.ml\(^{-1}\) the extract was considered to have low activity. Based on this, the secondary metabolites from THB-14 and ACT-20 are categorized as strains with very good antiplasmodial activity.

Maskey et al. (2004b) reported that, trioxacarcin-A showed IC\(_{50}\) 1.6 ng.ml\(^{-1}\) against *Plasmodium falciparum* K1 and *P. falciparum* NF54 isolated from *Streptomyces* sp. B8652. The salinosporamide A isolated from the actinomycetes *Salinospora tropica* showed IC\(_{50}\) 11.4 nM against *P. falciparum* (Jacques Pruchomme et al., 2008). The bioactive compounds from the marine fungus *Halorosellinia oceanica* BC 5149 showed IC\(_{50}\) 8.2 mM (Chinworrungetee et al., 2001). Isaka et al. (2007) reported that, the pullularin A from the endophytic fungus *Pullularia sp.* BCC 8613 exhibited antiplasmodial activity against the *Plasmodium falciparum* K1 and it showed
IC$_{50}$ value at 3.6 µg.ml$^{-1}$. Roger Linington et al. (2007) reported that, the marine cyanobacterium Oscillatoria sp. showed antiplasmodial activity with the IC$_{50}$ values of 8.2 and 5.2 µM. Roger Linington et al. (2009) reported that gallinamide A showed antiplasmodial activity against the chloroquine resistant Plasmodium falciparum W2 and showed IC$_{50}$ at 8.4 µM.

The most promising strains isolated from the sponges (ACT-20 and THB-14) which showed maximum inhibition of parasitaemia were subjected for species identification through 16S rRNA sequencing as a part of molecular standardization of drugs of biological origin. 16S rRNA sequencing analysis has been widely applied for the study of the diversity of microbial community and for strain identification. The present study also made an attempt to find out the most promising antiplasmodial strains of sponge associated microbes through 16S rRNA sequencing. The phylogenetic analysis of 16S rRNA gene sequences revealed that, the ACT-20 strain belonged to Streptomyces sp. and named as Streptomyces sp. RJAUACT-20 (GU269570) and comes under the family of Streptomycetaceae. Streptomyces is widely distributed in nature and found to be the good source of commercial enzymes and therapeutically useful bioactive molecules (Stach and Bull, 2005). Earlier investigation reveals that, Streptomyces strains isolated from marine environments (Colquhoun et al., 1998) have many novel bioactive compounds with several unique structures (Lee et al., 1998;
Itoh et al., 2003; Sanchez Lopez et al., 2003). Maldonado et al. (2005) reported that, the *Micromonospora, Rhodococcus* and *Streptomyces* group is the dominant, ubiquitous actinobacteria in marine environments.

Zhang et al. (2006) reported that, the phylogenetic analysis using 16S rRNA gene sequences revealed that the isolates belonged to seven genera of culturable actinobacteria including *Actinoalloteichus, Micromonospora, Nocardia, Nocardipsis, Pseudonocardia, Rhodococcus* and *Streptomyces*. The dominant genus was *Streptomyces*, which represented 74% of the isolates. The strains of *Brachybacterium, Gordonia, Micrococcus* and *Streptomyces* were isolated from the sponges *Xestospongia muta, R. odorabile* and other unidentified sponges (Bultel Ponce et al., 1998; Lee et al., 1998; Montalvo et al., 2005).

Jiang et al. (2008) find out that, the phylogenetic analysis based on 16S rRNA gene sequencing from marine sponge *Iotrochota sp.* isolates belonged to the genera *Streptomyces, Cellulosimicrobium* and *Nocardiopsis sp.* the majority of the strains belonged to the genus *Streptomyces*. Jiang et al. (2007) reported that, the phylogenetic analysis of culturable actinobacteria isolated from the marine sponge *Haliclona sp.* belonged to the genera *Streptomyces, Nocardiopsis, Micromonospora* and *Verrucosispora* by using 16S rRNA gene sequencing.
In silico analysis shows that, the strain THB-14 was identified as Bacterium RJAUTHB-14 comes under the family of Enterobacteriaceae. Muscholl Silberborn et al. (2007) reported that, the sponge associated most abundant bacteria were all affiliated to the Alphaproteobacteria and identified by using 16S rDNA sequencing. Radjasa et al. (2007) noted that, the richness of secondary metabolite producing sponge associated bacteria having closest similarity to Vibrio parahaemolyticus, Pseudovibrio denitrificans, Pseudoalteromonas sp. and Alphaproteobacterium by using 16S rRNA gene sequencing. Ganesh Babu et al. (2004) reported that, the sequence analysis of 4 isolates belong to Bacillus sp. and the 5th one being Enterobacter cloacae isolated from coral reef ecosystem of Gulf of Mannar Marine Biosphere Reserve using 16S rRNA sequencing. Thiel et al. (2007) noted that, the stable and specific bacterial community associated with the Mediterranean sponge Chondrilla nucula belonging to the Acidobacteria, Gammaproteobacteria and Deltaproteobacteria. Longford et al. (2007) reported that, the bacterial communities associated with the demosponge Cymbastela concentrica by using 16S rRNA sequencing and the isolates are belonged to Proteobacteria, Actinobacteria and Nitrospira.
As a part of biological standardization of newer drugs developed from the biological resources, the present study made an attempt to evaluate the toxicity of crude extracts if any obtained from Bacterium RJAUTHB-14 and *Streptomyces sp.* RJAUACT-20 strains. In the present study, the ethyl acetate extract of Bacterium RJAUTHB-14 and *Streptomyces sp.* RJAUACT-20 were found to be non toxic up to the dose level of 2500 and 3000 µg.kg⁻¹ bw in rats when administered orally. There were no changes in animal behavior, body weight and organ weight at all doses of the treated rats, when compare to the control group.

Moreover, no changes have been noticed in serum parameters such as AST, ALT, ALP, protein, albumin, globulin and bilirubin. AST, ALT and ALP are well known enzyme used as biomarkers predicting possible toxicity to the parenchymal liver cells (Rahman *et al.*, 2001). The blood parameters such as polymorphonuclear leucocytes, lymphocytes, eosinophils, RBC, PCV%, platelet, cholesterol, TGL, HDL, LDL, VLDL, sugar and urea showed negligible changes. The increase in the level of WBC and haemoglobin might be due to the boosting of immune system of treated groups. The significance decrease in blood glucose level by the administration of crude extract obtained from Bacterium RJAUTHB-14 treated rats might be due to the presence of hypoglycaemic components. Histopathological examination of the liver, kidney, heart, lungs and testis did not showed any abnormalities in
both the extracts obtained from Bacterium RJAUTHB-14 and Streptomyces sp. RJAUACT-20. Hence, the bioactive crude extracts obtained from two microbial strains are considered as safe and effective antiplasmodial agents.

Standardization of a drug is a part of drug development. As a part of the standardization, the present study also made an attempt to find out the organoleptic and physical properties of crude extracts from the most promising antiplasmodial strains Bacterium RJAUTHB-14 and Streptomyces sp. RJAUACT-20. The organoleptic properties such as taste, colour, odour, consistency and the physical properties such as total ash content, acid insoluble ash, water soluble ash were found within the acceptable limits as the WHO guidelines (WHO, 2000). The marine microorganisms have proved as rich source of compounds that might be useful for the development of new pharmaceutical agent. Modern pharmacognosy has greatly changed and gradually advanced towards the chemical aspects of the drug which include both qualitative and quantitative analysis of its constituents and other contaminants. It was already reported that, marine microorganisms in contaminated and high salt environment known to accumulate a variety of heavy metal contaminants derived from adjacent drainages. When potential crude extracts were identified for the future development of drugs, the presence of such contaminants could bring side effects rather than the curative actions. Hence, the present study has made an attempt to find out
the dangerous heavy metals found in efficient antagonistic sponge associated actinomycetes. The results reveals that, all the metals are within the safer levels of heavy metal contaminants as per the acceptable limits of WHO guidelines (WHO, 2000).

The present study also reveals that, there are no potential pathogenic contaminants in the crude extracts of most potential antiplasmodial strains Bacterium RJAUTHB-14 and Streptomyces sp. RJAUACT-20 and hence, the crude extracts obtained from the sponge associated Bacterium RJAUTHB-14 and Streptomyces sp. RJAUACT-20 are found to be safe and efficacious drugs for the management of malaria after completing successful clinical trails.