5. DISCUSSION

Plants are an important source of disinfection compounds as they produce a wide range of phytochemicals with antimicrobial properties, most of them against microorganisms, insects, nematodes and other plants (Abreu et al., 2012). Phytochemicals are able to inhibit peptidoglycan synthesis, damage microbial membrane structures modify bacterial membrane surface hydrophobicity and also modulate quorum-sensing (Rasooli et al., 2008).

Plants are rich in a wide variety of secondary metabolites such as tannins, alkaloids, steroids, terpenoids and flavonoids, which have been found in vitro to have antimicrobial properties (Lewis and Ausubel, 2006). A number of phytotherapy manuals have been mentioned various medicinal plants for treating infectious diseases due to their availability, fewer side effects and reduced toxicity (Lee et al., 2007). In recent years, the development of microbial resistance to common antibiotics due to indiscriminate use of commercial antibiotics forced researchers to search for novel antimicrobial substances from various sources. (Blunt et al., 2007).

Marine macro algae have been used to targeted antimicrobial chemical defence strategies and secondary metabolites are important in the ecological interactions between marine macroorganisms and microorganisms. Therefore, they could be a promising source of novel
bioactive compounds. Several metabolites with unusual structures have been isolated from the marine macroalgae and some of these metabolites are known to exhibit high order biological activities (Blunt et al., 2006).

The antimicrobial activities of the macroalgae have been attributed to the presence of biologically active compounds with antibacterial potential, such as cycloeudesmol, lyengaroside A, meroditerpenoid, neoirietetraol, diterpene-benzoate, polybrominated indoles, halogenated sesquiterpene alcohol, lanosol enol ether, diterpenebenzoic acids, callophycoic acids, halogenated diterpene-phenols, callophycols and eicosanoids (EI Gamal, 2010).

Mosquito bites may also cause allergic responses including local skin reactions and systemic reactions such as urticarial. Personal protection is one approach to prevent mosquito bites (Senthilkumar and Venkatesalu, 2012; Sakulku et al., 2009). Most common mosquito repellents available contain N,N-diethyl-3-methylbenzamide or also called DEET that has shown strong protection from mosquitoes. However, it may exert toxic reaction under some circumstances and age groups and damage plastic, synthetic materials, thus the alternative new products need to be explored (Revay et al., 2012; Chio and Yang, 2008).
The results on antimicrobial and mosquito larvicidal activities of Caulerpa chemnitzia, C. racemosa, C. scalpelliformis, Ulva lactuca, U. fasciata, U. reticulata, Stoechospermum marginatum, Sargassum wightii, Gracilaria edulis and G. verrucosa have been discussed in this chapter.

5.1. YIELD OF DIFFERENT EXTRACTS OF MARINE MACRO ALGAE

In the present investigation, the yield of different crude extracts ranged between 0.04 and 6.50 per cent for Caulerpa chemnitzia, C. racemosa, C. scalpelliformis, Ulva lactuca, U. fasciata, U. reticulata, Stoechospermum marginatum, Sargassum wightii, Gracilaria edulis and G. verrucosa. The successive isolation of botanical compounds from the plant materials is largely dependent upon the type of solvent used in the extraction procedure. Elnabris et al. (2013) reported that the U. lactuca yielded maximum extractable matter (17%), followed by Enteromorpha compressa (7.2%), Padina pavonica (5.2%) and Jania rubens (1.2%). Factors like the age of the plant and the polarity of the solvent has used affect the yield. Nanthini Devi et al. (2014) investigated that the yield per centage of extracts of Sargassum wightii was 15.2, 14.3 and 12.8 in ethanol, acetone and methanol, respectively. This difference in results may be due to difference in species used, time and place of sample collection, secondly; there may also be differences in the capability of the extraction protocols to recover the active metabolites.
5.2. ANTIMICROBIAL ACTIVITY OF CRUDE EXTRACTS OF SELECTED MARINE MACRO ALGAE

In the present study, the different extracts of hexane, chloroform, ethyl acetate, acetone and methanol extracts of *Caulerpa chemnitzia*, *C. racemosa*, *C. scalpelliformis*, *Ulva lactuca*, *U. fasciata*, *U. reticulata*, *Stoechospermum marginatum*, *Sargassum wightii*, *Gracilaria edulis* and *G. verrucosa* were screened for their antimicrobial activity against four Gram-positive bacteria *viz.*, *Bacillus subtilis*, *Enterococcus faecalis*, *Staphylococcus aureus* (MTCC 737 and 7443) and five strains of Gram-negative bacteria *viz.*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Shigella flexneri* and *Vibrio cholerae* and six yeast *viz.*, *Candida albicans*, *C. glabrata*, *C. guilliermondii*, *C. krusei*, *C. parapsilosis*, *C. tropicalis* and four dermatophytes *viz.*, *Epidermophyton floccosum*, *Microsporum gypseum*, *Trichophyton mentagrophytes*, *T. rubrum* and one isolate of MRSA. All the tested plant extracts showed the varied level of antibacterial and antifungal activities against tested bacterial and fungal strains. The mean zones of inhibition produced by all the algal extracts tested were ranged between 7.0 and 21.5 mm, and the lowest MIC values were between 62.5 and 500 µg/mL and MBC values were from 125 to 1000 µg/mL. The basis of varying degree of sensitivity of bacteria may be due to varied efflux pump availability in different strains and the nature and combination of phytocompounds present in crude extracts (Seasotiya and Dalal, 2014). Deris *et al.* (2014) reported that the mode of action of
the plant extracts against the specific bacteria may be due to its secondary mode of action against the bacterial enzymes instead of acting on the cell wall of the bacteria. Over the last decades, several studies have been reported that the antibacterial activity of marine algal extracts. Although the majority of these studies indicated variable activities against tested microorganisms, however, it is difficult to compare the results from these studies because the antimicrobial activity of algal extracts may be influenced by a number of factors including algal species (Chen et al., 2009), extraction methods, testing methodology, solvent used in extractions (Karthikaidevi et al., 2009; Tuney et al., 2006), season or time at which samples were collected (Marechal et al., 2004; Salvador et al., 2007), place of sample collection (Salvador et al., 2007) and the thallus regions used for extraction (Freile-Pelegrin and Morales, 2004).

In the present study, among the various extracts used to test the antimicrobial activity, the ethyl acetate extract obtained from Stoechospermum marginatum showed the highest mean zone of inhibition of 21.5 mm at 500 µg/disc against Staphylococcus aureus (7443). Thillairajasekar et al. (2009) reported that the ethyl acetate extracts of Ulva lactuca and Gracilaria verrucosa showed the highest antimicrobial activity against Escherichia coli, Klebsiella pneumoniae, MRSA and Bacillus subtilis and also identified the presence of myristic and palmitic acid, linoleic acid, oleic acid, lauric, stearic and myristic acid,
from ethyl acetate extracts. Salem et al. (2011) reported that the higher antibacterial activity was recorded for the ethyl acetate extracts of Caulerpa racemosa, Sargassum dentifolium, Padina gymnospora; methanol extracts of Sargassum hystrix, S. dentifolium, Caulerpa racemosa, Codium fragile and Cystoseria myrica. The fatty acid methyl ester extract of Stoechospermum marginatum showed the presence of myristic acid, palmitic acid, stearic acid, oleic acid, linoleic acid and linolenic acid (Venkatesalu et al., 2005). Fatty acid penetrates the microbial cell membrane and the enzyme activity inhibition (Schaafsma, 1996).

In the present study, the different extracts of all the tested marine macro algae showed potential anti MRSA activity. Similar observation Kim et al. (2007) had screened hexane, chloroform, ethyl acetate and methanol extracts of Ulva lactuca showed the antimicrobial activity against Bacillus subtilis, Micrococcus luteus, Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella typhimurium, Candida albicans and three MRSA strains. The results revealed that the highest mean zones of inhibition were recorded with the ethyl acetate extract against B. subtilis (15 mm), M. luteus (12 mm), S. aureus (18 mm), E. coli (17 mm), K. pneumoniae (8 mm), P. aeruginosa (8 mm), S. typhimurium (18 mm), Vibrio parahemolytics (18 mm), E. tarda (18 mm) and three MRSA strains with the mean zones of inhibition ranged between 18.0 and 27.0 mm. Lee et al. (2008) reported that the ethyl acetate-soluble fraction of Ecklonia stolonifera
and *Ecklonia cava* exhibited the strongest anti-MRSA activity. Dieckol has been isolated from *Ecklonia stolonifera* and *E. cava* is a known antibacterial substance with activity against MRSA.

In this study, the different extracts of selected marine macro algae against bacterial and fungal strains tested, among these, *Staphylococcus aureus* (7443) was most susceptible to the ethyl acetate extract of *Stoechospermum marginatum* with the lowest MIC value of (62.5 µg/mL). Chandrasekaran *et al.* (2014b and 2014c) reported that the highest antibacterial activity were recorded in the brown alga, *S. marginatum* against MRSA and Vancomycin resistant *Enterococcus faecalis* in the ethyl acetate extracts when compared to other solvents extracts. Shanmughapriya *et al.* (2008) reported that the *S. marginatum* extracts inhibited the growth of multi drug resistant *Klebsiella pneumoniae, Proteus mirabilis, Micrococcus luteus, Escherichia coli* and *Enterococcus faecalis*. The extract of *S. marginatum* has also exhibited strong antifungal activity (Usmanghani and Shameel, 1986).

In the present study, among the solvent extracts tested, the ethyl acetate extracts of *Stoechospermum marginatum, Caulerpa racemosa* and *Gracilaria edulis* showed the highest mean zones of inhibition (21.5, 19.5 and 16.6 mm at 500 µg/disc respectively) against *Staphylococcus aureus* (7443). Vallinayagam *et al.* (2009) reported that the *Ulva lactuca* and *Gracilaria edulis* showed activity against human bacterial pathogens such as *S. aureus, Vibrio chloerae, Shigella dysentriae,*
S. boydii, Salmonella paratyphi, Pseudomonas aeruginosa and Klebsiella pneumoniae. The methanol and aqueous extracts of Gracilaria verrucosa, G. ferugusonii, G. verrucosa var., Hypnea musciformis, Enatiocladia prolifera and Gelidium species showed the broad spectrum of antimicrobial activity against Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Streptococcus aureus Salmonella typhi and Candida albicans (Adaikala Raj et al., 2012).

Arputha Bibiana et al. (2012) reported that the petroleum ether, diethyl ether, acetone and acetic acid extracts of Sargassum wightii and Kappaphyus alvarezii showed a good antibacterial and antifungal activities against Streptococcus pneumoniae, Bacillus cereus, B. subtilis, Vibrio cholerae V. parahaemolyticus, Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa and fungi, Microsporum gypsum, Aspergillus fumigatus, A niger, A. flavus and Trichophyton rubrum.

In the present study, the different solvents viz., hexane, chloroform, ethyl acetate, acetone and methanol were used to get the extracts of selected marine macro algae and the extracts were investigated for antimicrobial activity. Generally, both solvents and extraction procedure are important to consider when evaluating the antimicrobial effect of compounds from plants. The extraction of active compounds is highly depending on the polarity of the solvent because
polar compound is easily extracted using polar solvent (Goli et al., 2004). Thus, the solvent used for the extraction of bioactive compounds must be critically chosen because it will influence the quantity and quality of the final extract (Sinero et al., 2008). The ethyl acetate extracts of selected marine macro algae showed the potential inhibitory action on tested bacterial and fungal strains. The solvent ethyl acetate is known for its ability to isolate more antimicrobials from plants including tannins, polyphenols, terpenoids, saponins, quassinoids, lactones, flavones and phenones (Cowan, 1999).

Chandrasekaran et al. (2014b) reported that the highest antibacterial activity were recorded in selected marine macro algae against Vancomycin resistant *E. faecalis* in ethyl acetate extracts when compared to other solvent extracts. The antibacterial and antifungal activities of individual plant may be due to the presence of phytochemicals. Chandrasekaran et al. (2014d) reported that phytochemical analysis of various solvents extracts revealed that the presence of terpenoids, tannins, phenolic compounds and steroids in *Sargassum wightii*. Seaweeds provide a rich source of structurally diverse secondary metabolites. Several studies have demonstrated that seaweeds are an excellent source of components such as polysaccharides, tannins, flavonoids, phenolic acids, bromophenols and carotenoids which exhibit different biological activities (Rodriguez-Bernaldo de Quiros et al., 2010).
Many tannins containing drugs are used in medicine as astringent. They are used in the treatment of burns as they precipitate the proteins of exposed tissues to form a protective covering. They are also medicinally used as healing agents in inflammation, leucorrhoea, gonorrhoea, burns, piles and as antidote. Tannins have been found to be with antiviral, antibacterial, antiparasitic effects, anti-inflammatory, antiulcer and antioxidant properties for possible therapeutic applications (Kolodziej and Kiderlen, 2005). Steroids of plant origin are known to be important for insecticidal, antimicrobial, antiparasitic and cardiotonic properties. Steroids also play an important role in nutrition, herbal medicine and cosmetics (Okwu, 2001).

Phenolic compounds are commonly found in plants, including seaweeds and have been reported to have a wide range of biological activities including antioxidant properties (Duan et al., 2006), antibacterial, antiviral, antifungal (Adekunle and Ikumapayi, 2006) and antiulcer activities (Kolodziej and Kiderlen, 2005). Phenolic compounds may affect growth and metabolism of bacteria. They could have an activating or inhibiting effect on microbial growth according to their constitution and concentration (Reguant et al., 2000). Zapata and McMillan (1979) reported that the role of phenolic compounds present in seagrasses could also enhance the antimicrobial activity. This may be due to active components which are present in the
seaweed extracts. These results indicate that the extracts contained different antibacterial substances and reflect the variety of secondary metabolites.

In the present study, Gram-positive bacteria were more susceptible than the Gram-negative bacteria for the tested extract and compounds of marine macro algal species.

Taskin et al. (2001) reported that the similar observations, indicating that the more susceptibility of Gram-positive bacteria to the algal crude extract was due to the differences in their cell wall structure and their composition (Paz et al., 1995). The resistance of Gram-negative bacteria towards antibacterial substances is related to the hydrophilic surface of their outer membrane, which is rich in lipopolysaccharide molecules, presenting a barrier to the penetration of numerous antibiotic molecules. The membrane is also associated with the enzymes in the periplasmic space which are capable of breaking down the molecules introduced from outside (Shan et al., 2007). However, the Gram-positive bacteria do not possess such outer membrane and cell wall structures (Kalamba and Kanicka, 2003). In the case of Gram-negative bacterial strains, thin layer of peptidoglycan in the cell may fail to provide an active site for the binding of the bioactive compound leading to reduced inhibitory effect. The concentration at which the extracts have been loaded in the discs may also contribute
to its inhibitory action. The importance of defensins in innate immunity of humans is underscored by the observation that certain disorders characterized by recurrent infections associated with a lack of defensins in blood phagocytes (Ganz et al., 1988). Michele et al. (1997) suggested that the absence of target structure in bacteria and the ability of bacteria to alter the structure and genetic changes in bacteria lead to the alteration of metabolic pathway that is originally blocked by the antibacterial agents. Beyond all the environmental factors affecting the resistance explained before, there are cellular mechanisms influencing this process (McDonnell and Russell, 1999).

Theanphong et al. (2008) reported that the essential oil tested had strong inhibitory activity against Gram-positive bacteria (Staphylococcus aureus, Bacillus subtilis and Micrococcus luteus) but it showed the weak activity against Gram-negative bacteria (E. coli). The greater resistances of Gram-negative bacteria to plant extracts have been documented previously for seeds of Syzygium jambolanum (Chandrasekaran and Venkatesalu, 2004).

In this study, antibacterial drugs viz., Methicillin for S. aureus and MRSA, Vancomycin for E. faecalis and Ampicillin and Ciprofloxacin for all other bacterial strains tested were used as positive controls. In India, the epidemiology of MRSA is changing over the past few decades. The resistance of MRSA to β-lactams like Penicillin and Amoxicillin was 100% (Vidhani et al., 2001). Vancomycin, a tricyclic glycopeptides
antibiotic, is used to treat MRSA infections (Lee et al., 2007). Vancomycin interferes with bacterial cell wall synthesis, as does Penicillin, eventually leading to cell lysis (Barna and Williams, 1984). Thus, the development of new drugs or alternative therapies is clearly a matter of urgency (Hiramatsu et al., 1997). Topical drugs such as mupirocin and fusidic acids are still effective against certain strains of MRSA, but resistance is increasing against these drugs as well selection of appropriate antibiotics for MRSA infection, dosage and side effect (Howden and Grayson, 2006).

Resistance by a microorganism to a drug means that normal dose of the drug becomes ineffective in treating the infection. Because every drug has both the desired effects and adverse side effects, attempts to increase doses of drugs to make them have effects on resistant pathogens and make the adverse effects of the drugs become more prominent than their desired effects. So, the overall effects of the treatment become toxic. Apart from direct interference with physiologic functions of the body, toxic effects of some drugs, including Ampicillin, lead to immune suppression (NTP, 1987). When a treatment fails to inhibit causative agent of an infectious disease but rather causes immune suppression, the infection rate increases, because, the patient’s immunity which was keeping it in check would have been compromised, without a corresponding level of effect on the pathogen. Since toxic effects of Ampicillin occur only at high doses
NTP (1987), an increase in activity of its lower doses would make its desired effect (inhibition of disease causing agents) more prominent than its toxic effects. The search for new ways to treat bacterial infections stimulates the investigation of natural compounds have an alternative treatment of these infections.

In our study, we are using two standard antifungal drugs like Amphotericin-B for yeast and Ketoconazole for dermatophytes. Amphotericin-B is considered as the drug of choice for the treatment of fungal infections (Hartsel and Weiland, 2003). However, toxicity and resistance to these antifungal drugs are a major problem (Edwards, 1991). In case of Amphotericin-B, due to its poor permeability across the membrane (Matsuoka and Murata, 2002) an increased amount of Amphotericin-B must be administered to patients, which can result in severe side effects such as renal damage (Mayer et al., 2002). To lessen the severity of side effects, Amphotericin-B is often combined with other antifungal drugs such as the azoles (Lewis et al., 1998). Ketoconazole is an imidazole antifungal agent currently used in the treatment of a broad range of fungal infections; the drug inhibits cytochrome P450 14a-demthylase, an enzyme involved in the synthesis of ergosterol, a crucial component the fungal cell wall (Gunt and Kasting, 2007). However, the unpleasant side effects of this drug include nausea, abdominal pain, itching and its toxicity limits its therapeutic use in many cases (Sugar et al., 1987).
5.3. ANTIBACTERIAL ACTIVITY OF ISOLATED COMPOUNDS Sm1, Ca1, Ca2 AND Ge FROM SELECTED MARINE MACRO ALGAE

The present study, the active compound, 3′H-cycloprop-(1,2)-5.alpha.-cholest-1-en-3-one, 1′,1′-dicarboethoxy-1.beta.,2.beta.-dihydro-,diethyl6-(1,5-dimethylhexyl)-3b,5a-dimethyl-2-oxohexadecahydro-cyclopenta[a]cyclopropa-[g]phenanthrene-3,3(1H)-dicarboxylate (Sm1, a Keto-steroidyl diester) was isolated and identified from the ethyl acetate extract of Stoechospermum marginatum.

The phytochemicals present in plants are responsible for preventing disease and promoting has been studied extensively to establish their efficacy and to understand the underlying mechanism and their action. Such studies have included identification and isolation of the chemical components, establishment of their biological potency both by in vitro and in vivo studies in experimental animals and through epidemiological and clinical-case control studies in man (Mathai, 2000).

Steroids have been the important focus of research throughout the scientific history. But the recent past has seen an exhaustive focus of research being diverted towards these biologically important molecules. These compounds turn out to be non-toxic, less vulnerable to multi-drug resistance (MDR) and highly bioavailable because of being capable of penetrating the cell wall (Bandey et al., 2011).
Steroid based antimicrobial agents continue to play a prominent role in those organisms which do not rely upon external supply of drugs to fight against pathogens (Savage, 2002) because the entire morbidity and mortality mostly in developing countries is due to these microbial infections (Qadri et al., 2005) among which *Escherichia coli* is responsible for the most common and serious infectious diseases like invasive dysentery and diarrhoea (Zhang et al., 2006).

Phytochemical and pharmacological studies have been undertaken in order to revealed that the presence of steroids with antimicrobial activity. These reports mainly concerned their antifungal activity. Eurysterols A and B are two new steroidal sulfates isolated from an undescribed marine sponge of the genus *Euryspongia* (Boonlarppradab and Faulkner, 2007). Bioassay-guided fractionation of the extract of *Topsentia* sp. led to the identification of two new sulfated sterols, geodisterol-3-O-sulfite and 29-demethylgeodisterol-3-O-sulfite, as active constituents reversing efflux pump mediated Fluconazole resistance (Digirolamo et al., 2009).

Wang et al. (2013) described the cytotoxic effects of 14 new polyoxygenated steroids from the gorgonian *Menella kanisa*. These new steroids were active against lung (A549) and osteosarcoma (MG-63) cell lines. Related studies of antimicrobial activity indicate that the crude extracts containing flavonoids, triterpenes and steroids against various strains of *Staphylococcus aureus*, *Streptococcus faecalis* and *Escherichia coli* (Chattopadhyay et al., 2001).
5.3.1. 3'H-cycloprop(1,2)-5.alpha.-cholest-1-en-3-one, 1',1'-dicarboethoxy-1.beta.,2.beta.-dihydro-,diethyl6-(1,5-dimethylhexyl)-3b,5a-dimethyl-2-oxohexadecahydrocyclopenta[a]cyclopropa-[g]phenanthrene-3,3(1H)-dicarboxylate (Sm1, a Keto-steroidyl diester)

The compound, Keto-steroidyl diester possessed antibacterial activity against tested bacterial strains. The mean zones of inhibition were ranged between 9.3 and 27.1 mm against all tested bacterial strains. The lowest MIC values were between 12.5 and 50 µg/mL, while the MBC values were between 25 and 100 µg/mL were recorded against B. subtilis, MRSA and S. aureus (7443 and 737). The highest mean of zone inhibition (27.1 mm) was observed with the isolated compound, Sm1 against S. aureus (MTCC 7443).

A new spatane diterpene, 17,18-epoxy,5(R),16-dihydroxyspata13(14)-ene, was isolated from a brown alga, Stoechospermum marginatum (Venkateswarlu and Biabani, 1995) and various bioactivities, including antibacterial and antifungal activities.

Shiaikh et al. (1990) reported that the sterols and diterpenes were isolated from the chloroform and methanol extracts of S. marginatum such as cholesterol, 24-methylene cholesterol and 24-methyl cholesterol and diterpenes viz., 19-acetoxy-5(R),15,18(R and S)-tetrahydroxyspata-13,16(E)-diene, 5(R),15,18 (R and S),19-tetrahydrowspata-13,16 (E)-diene, 5 (R),18-dihydroxyspata-13,16 (E)-dime,
5(R),16-dihydroxyspata-13,17-diene and D-mannitol. All these diterpenes and sterols showed the strong antibacterial activity against three Gram-positive and six Gram-negative bacteria. The active constituents were found to be a mixture of monoacetates belonging to the spatane diterpenoids, the structures of which have been elucidated by Gerwick et al. (1981).

De Almedia et al. (2011) reported that the cholesterol and clinoasterol present in Gracilaria crassa, G. coronopifolia, G. longa and G. dura. Other steroids such as 3-beta-hydroxy-poriferast-5-en-7-one, 3-beta-7-alpha-diol-poriferast-5-ENE and 5-alpha-poriferast-9(11)-en-3-beta-ol were isolated from G. dura; cholestane-3-5-diol,5:24(S)-ethyl, poriferastene 8, poriferast-5-ene-3-7-diol and poriferast-5-ene-3-7-diol were isolated and identified in G. coronopifolia and G. longa also has various compounds like alpha linolenic acid, gamma linolenic acid, glycolipids, 5-dehydro avenasterol, fucosterol, myristic acid, desmosterol and 5-alpha-24(S)-ethyl-cholestane-3-beta-6-beta-diol. Steroids are apparently involved in the regulation of large number of biological activities including electrolytic and hormonal balance as well as reaction to allergy. Steroids have anti-inflammatory and antioxidant activities (Solomons, 1998).
5.3.2. 2,7-Diphenyl-1,6-dioxopyridazino[4,5:2′,3′]pyrrolo[4′,5′-d]pyridazine (Ca1, an alkaloid)

In the present study, the active compound, 2,7-diphenyl-1,6-dioxopyridazino[4,5:2′,3′]pyrrolo[4′,5′-d]pyridazine (Ca1 an alkaloid) was isolated and identified from the ethyl acetate extract of Caulerpa racemosa.

Alkaloid is a group of biological amine and cyclic compounds having nitrogen in the ring, naturally occurring in plants, microbes, animals and marine organisms. Cyclic nitrogen compounds in halogenated form are predominantly found in marine organisms and algae. Both halogenated and non-halogenated forms have attracted researchers’ interest because of their pharmaceutical importance as bioactive compounds and as biological probes for physiological studies (Kasim et al., 2010).

The isolated compound, 2,7-diphenyl-1,6-dioxopyridazino[4,5:2′,3′]-pyrrolo[4′,5′-d]pyridazine (Ca1) was screened for their antibacterial activity against all the bacterial strains tested. The mean zones of inhibition produced by Ca1 against all the bacterial strains tested that ranged between 8.5 and 23.5 mm. The result of MIC values of the tested compound ranged between 12.5 and 50 µg/mL, while the MBC values were between 25 and 100 µg/mL were recorded against B. subtilis, MRSA and S. aureus (7443 and 737). The highest mean of zone inhibition (21.3 mm) was observed with isolated compound, Ca1 against S. aureus (MTCC 7443).
Caulerpin is the only reported alkaloid from seaweed with anti-inflammatory activity. Caulerpin, a bisindole alkaloid because it contains 2 indole groups (benzylpyrrole derived from tryptophan) linked together by 8 carbons cyclic ring with two carboxyl groups (Kasim et al., 2010). Caulerpin has been isolated mainly from green and red algae. Isolation of Caulerpin (CLP) from seaweed (Caulerpa sp.) was first conducted as far back as 1970 and tagged as CLP I (Aguilar-Santos, 1970), 21 years later other analogues were isolated from Caulerpa racemosa and referred to as CLP II and CLP III (Anjaneyulu et al., 1991) while in 1994 crystal structure of CLP I was determined by Lu et al. (1994).

The genus Caulerpa has been widely studied and the structures of many new compounds, such as di-, sesqui- and mono-terpenes with the terminal 1,4-diacetoxybutadiene moiety and the nitrogen-containing compounds bisindole alkaloids (exemplified by caulerpin) and caulerpicin Mao et al. (2006). Recently, the genus Caulerpa has attracted the attention of researchers due to its important secondary metabolite caulerpenyne (CYN) that is reported to exhibit the antineoplastic, antibacterial and antiproliferative activities (Barbier et al., 2001; Cavas et al., 2006). Alkaloids, such as berberine and piperine, interact with bacterial cytoplasmic membrane, intercalate with DNA or inhibit efflux pumps in Staphylococcus aureus (Khan et al., 2006).
5.3.3. Z,Z-6,28-Heptatriacontadien-2-one (Ca2, a fatty acid)

In the present study, the active compound, Z,Z-6,28-heptatriacontadien-2-one (Ca2, a fatty acid) was isolated and identified from the ethyl acetate extract of Caulerpa racemosa.

Fatty acids are carbon chains with a methyl group at one end of the molecule (designated omega, \(\omega\)) and a carboxyl group at the other end. The carbon atom next to the carboxyl group is called \(\alpha\) carbon and the subsequent one, the \(\beta\)-carbon. The letter \(n\) is also often used instead of the Greek \(\omega\) to indicate the position of the double bond closest to the methyl end. The systematic nomenclature for fatty acids may also indicate the location of double bonds with reference to the carboxyl group (\(\Delta\)) (Rustan and Drevon, 2005).

Fatty acids and their corresponding esters are one group of chemicals found in nature considered to have little or no toxicity, with proven antimicrobial activity and it showed that fatty acids esterified with monohydric alcohols were inactive against microorganisms, those esterified with certain polyhydric alcohols yielded antimicrobial derivatives (Kabara et al., 1972; Conley and Kabara, 1973). Fatty acids are widely occurring in natural fats and dietary oils and they play an important role as nutritious substances and metabolites in living organisms (Cakir, 2004). Many fatty acids are known to have antibacterial
and antifungal properties (Russel, 1991). Recently, a number of novel fatty acid derivatives of carbohydrates have been synthesized and their antimicrobial activity has been assessed (Devulapalle et al., 2004; Ferrer et al., 2005). Generally, long-chain fatty acids have activity against Gram-positive bacteria, while short-chain fatty acids are more active against Gram-negative bacteria. Lauric acid (a medium-chain fatty acid) is regarded as the most active, with reported activity against both Gram-positive and Gram-negative bacteria (Kabara, 1983).

The compound, Z,Z-6,28-heptatriacontadien-2-one Ca2 also possessed differential effect against tested pathogens. The mean zones of inhibition ranged between 8.0 and 19.8 mm. The MIC values were between 25 and 100 µg/mL, while the MBC values were between 50 and 100 µg/mL were recorded against B. subtilis, MRSA and S. aureus (7443 and 737). The highest mean of zone inhibition (19.8 mm) was observed with the isolated compound against S. aureus (MTCC 7443).

Previous phytochemical investigations on Caulerpa racemosa has been presence of sesquiterpenes such as caulerpenyne (Anjaneyulu et al., 1991a; Dumay et al., 2002; Alarif et al., 2010), diterpenes such as cis- and trans-phytol (Capon et al., 1983; Anjaneyulu et al., 1991b; Alarif et al., 2010), three red pigments (caulerpin, caulerpinic acid and monomethyl caulerpinate)
(Anjaneyulu et al., 1991a,b, 1992), sulfated polysaccharides (Rodrigues et al., 2011), sterols (Anjaneyulu et al., 1991b; Aknin et al., 1992), a mixture of ceramides from (2S,3R)-sphinganine (Nielsen et al., 1982), a sulfoquinovosyldiacylglycerol (Wang et al., 2007) and aromatic derivatives (Anjaneyulu et al., 1992).

Rahul et al. (2014) reported that the Caulerpa racemosa showed the analyses of major constituents like pseudoephedrine, 5-butyl-2-methyl-δ1-pyrrolidine, 2-myristyno-yl pantetheine, tetratetracontane and deoxy-spergualin, hexyl octyl ether, etc. Recently pseudoephedrine has been reported to elicit a potent anti-inflammatory activity against acute liver failure model in rats, and this comprehensive anti-inflammatory effect may result from the inhibition of TNF-α production (Wu et al., 2014). Pyrrolidine class of compound has shown the protective effect on islet β-cells from oxidative damage and improves insulin production in a diabetic rat model (Ding et al., 2014). The antioxidant and cytoprotective activities have also been reported for tetratetracontane (Ertas et al., 2014) and deoxyspergualin (Matsui et al., 2002). In a post-marketing surveillance study, Donati et al. (1989) have recommended pantethine therapy for the treatment of lipid abnormalities also in patients at risk such as those with diabetes mellitus. Further, hexyl octyl ether class of compound has been reported to possess cytoprotective activity (Munoz-Marín et al., 2012).
5.3.4. 6,10,14-Trimethyl-2-pentadecanone (*Ge*, a fatty acid)

In the present study, the active compound, 6,10,14-trimethyl-2-pentadecanone (*Ge*, a fatty acid) was isolated and identified from the ethyl acetate extract of *Gracilaria edulis*.

Fatty acids are hydrocarbon chains with carboxyl group at the head end and a methyl group at the tail end. The carbons may be connected by single or double bonds. Fatty acids are important for human and animal health because they are precursor in the biosynthesis of eicosanoids, which are important bioregulators in many cellular process (Gressler *et al.*, 2010). It was also reported that the fatty acid of certain seaweeds have anticancer (Harada *et al.*, 2002), anti-inflammatory (Jaswir and Mansur, 2011), antibacterial (Shahnaz and Shameel, 2007) and antimicrobial (Sanaa, 2007) activities.

The major component in all analyzed species is the methyl ester of the saturated fatty acid hexadecanoic acid (palmitic acid) which is exhibited in high percentage in *Gracilaria birdiae* (41.86%), *G. caudata* (21.34%), *G. cerviconis* (50.94%) and *G. domingensis* (13.46%). *Gracilaria* has as the highest amount of saturated fatty acid and palmitic acid (Gressler *et al.*, 2010; Wen *et al.*, 2006). In addition, evidence on literature has shown that palmitic acid isolated from a marine red alga may be a lead compound of anticancer drugs (Harada *et al.*, 2002).
The isolated compound, 6,10,14-trimethyl-2-pentadecanone (Ge a fatty acid) was screened for their and antibacterial activity against all the bacterial strains tested. The mean zones of inhibition that ranged were between 8.0 and 19.5 mm. The result of MIC values of the tested compound ranged between 25 and 50 µg/mL, while the MBC values were between 50 and 100 µg/mL. The highest mean zone of inhibition (19.5±0.76 mm) of this compound was recorded against *S. aureus* (7443), the lowest MIC (25 µg/mL) and MBC (50 µg/mL) values were recorded against *B. subtilis*, MRSA and *S. aureus* (7443 and 737).

The chemical composition of the total methyl esters of fatty acids from the ethyl acetate extracts of *Gracilaria birdiae*, *G. caudata*, *G. cerviconis* and *G. domingensis* showed to have very similar profile. The hydrocarbon heptadecane together with the methyl esters of tetradecanoic, 2-pentadecanone, 6,10,14-trimethyl, 9-octadecenoic, octadecanoic and hexadecanoic acids were identified in all extracts (Anna *et al.*, 2012). Plaza *et al.* (2010) reported that the antimicrobial activity has been more attributed to long-chain unsaturated fatty acids (C<sub>16</sub>-C<sub>20</sub>) such as oleic and linolenic acids, long-chain saturated fatty acids fatty acids, including palmitic and stearic acids are known to have the same effect.

Many mechanisms of antimicrobial action of phytochemicals have been suggested by different researchers (Manson, 2003), opined that phytochemicals may act by inhibiting microbial growth, inducing
cellular membrane perturbations, interference with certain microbial metabolic processes, modulation of signal transduction or gene expression pathways. Plant-based constituents may exhibit different modes of action against enterotoxigenic bacterial strains which range from interference with the phospholipoidal cell membranes, which has as a consequence of increasing the permeability profile and loss of cellular constituents, damage of the enzymes involved in the production of cellular energy and synthesis of structural components and destruction or inactivation of genetic material. In general, the mechanism of action is considered to be the disturbance of the cytoplasmic membrane, disrupting the proton motive force, electron flow, active transport mechanisms and coagulation of cell composition (Kotzekidou et al., 2008).

5.4. MOLECULAR MODELING OF THE ISOLATED ANTIBACTERIAL COMPOUNDS OF Sm1, Ca1, Ca2 AND Ge

Molecular modeling runs of all known co-crystallized bound ligands (Sm1 and Ca2) with 1HNJ (β-ketoacyl-acyl carrier protein synthase III) (ecKAS III) targeted proteins obtained from Protein Data Bank (http://www.pdb.org/pdb/home/home.do) which was resolved at 1.46 Å using X-ray diffraction. For validating the software, the proteins were docked with the already bound ligand (Cheng et al., 2009). The Schiff bases were reported as a potent inhibitor of Plasmodium falciparum KAS III (Prigge et al., 2003) and ecKAS III with antimicrobial activity against various bacteria (Lee et al., 2009b).
In that, the Sm1 and Ca2 compound has good glide score and energy compared to 1HNJ (the best glide score of Sm1 = −4.81 and Ca2 = −4.5 kcal mol\(^{-1}\)) and also confirmed the results with good binding energy. The best docked posses (with lowest Glide Score value) obtained from Glide (Friesner et al., 2004; Friesner et al., 2006) was analyzed. The acting force of this binding mode is mainly depends on hydrogen bonding, electrostatic forces, van der Waals forces and hydrophobic interaction due to non-polar residue interaction and water structure effect alteration. The glide scores are mainly used to identify the active and inactive molecules. Friesner et al. (2006) reported that the addition, glide is primarily concerned with generating an accurate pose for each molecule and enrichment (the separation of actives from inactives).

Molecular docking is a powerful tool in drug design, which could predict the best mode by which a given compound fits well into a binding site of a macromolecular target (Chen et al., 2006). With in vitro antimicrobial result in hand, we thought it worthwhile to perform in silico studies to support the result (Singh et al., 2012). The crystal structure of the C(30) carotenoids dehydrosqualene synthase from Staphylococcus aureus complexed with BPH-673 (PDB code: 3ACX, www.rcsb.org) was obtained from protein data bank in PDB format as starting point.

The results revealed that the obtained data, the tested compounds (Ca1 and Ge) have been promising scores of docking with enzymes related to bacterial infections, with energy score values of
\( \text{Ca1} = -10.2 \) and \( \text{Ge} = -7.4 \) kcal mol\(^{-1}\) also confirmed the result with good binding energy. The minimum Glide energy required for the formation of complex between ligand and the receptor indicates excellent binding affinity. Very low energy indicates that the ligand is buried in the cavity of the receptor (Daisy et al., 2012). Therefore, there is an urgent need to develop effective antibacterial compounds and also to identify the potent target to combat this infection. In the present study, a molecular docking is performed to explore antibacterial activity of compounds in \textit{Camellia sinensis} leaf extracts (Shimamura et al., 2007) against UPPS protein of \textit{S. aureus} subsp. \textit{aureus} N315 (Zhu et al., 2013).

Whereas, the cell wall of \textit{S. aureus} is composed of 30-50 peptidoglycan layers. It is a cross-link between polysaccharides and peptide complex, which provides rigidity of the bacterial cell wall and protect the cell lysis from osmotic pressure (Shimamura et al., 2007).

### 5.5. Larvicidal Activity of Selected Marine Macro Algae

Current strategies based on the elimination of breeding sites and applications of chemical insecticides for larval and adult mosquito control have resulted in development of resistance without eliminating the constant risk of dengue epidemics (Govindarajan et al., 2008). Thus new approaches are urgently needed. Interest on possible use of environment friendly natural products such as extracts of plants or plant parts increased for vector control. Plant derived products have
received increased attention from scientists and more than 2000 plant species are already known to have insecticide properties (Isman, 2001). Algae synthesize a number of chemically diversified secondary metabolites. Among them, some of the compounds are recognized as insecticides. The control of adult mosquito is an unsuccessful strategy, as the adult stage occurs beside human inhabitation and they can easily overcome remedial measures (Service, 1993).

In the present study, the results of larvicidal activity of marine macro algal species revealed that Stoechospermum marginatum possessed the highest larvicidal efficacy against Aedes aegypti (LC$_{50}$ = 549.6 and LC$_{90}$ = 1222.9 ppm values at 24 h and LC$_{50}$ and LC$_{90}$ values of 481.2 and 1138.7 ppm at 48 h) followed by Ulva lactua, Gracilaria edulis, Caulerpa racemosa, Sargassum wightii, G. verrucosa, U. fasciata, C. scalpelliformis, Ulva reticulata and C. chemnitzia. Thangam and Kathiresan (1991) reported that the acetone extracts of Caulerpa scalpelliformis, Dichotoma, Enteromorpha clathrata, E. intestinalis and U. lactuca were active against fourth instar larva of A. aegypti with LC$_{50}$ values of 53.70, 61.65, 85.11, 67.70 and 91.20 ppm, respectively. Manilal et al. (2011) reported that the methanol extracts of Lobophora variegata, Spatoglossum asperum and Stoechospermum marginatum were found to possess larvicidal activity with and LD$_{50}$ values of 95.5, 96.1 and 97.3 ppm against third instar larva of A. aegypti.
In the present study, larvicidal activity of the ethyl acetate extract of *Stoechospermum marginatum* showed the remarkable larvicidal activity against *Aedes aegypti*. (LC$_{50}$ = 549.6 and LC$_{90}$ = 1222.9 ppm values at 24 h and LC$_{50}$ and LC$_{90}$ values of 481.2 and 1138.7 ppm at 48 h. Mullai and Jebanesan (2007) reported that the ethyl acetate leaf extracts of *Citrullus colocynthis* and *Cucurbita maxima* had larvicidal activity. The extracts showed the LC$_{50}$ values of 47.58 and 75.91 ppm, respectively, against *C. quinquefasciatus* larvae. The results reported by Arivoli et al. (1999) showed the ethyl acetate extracts of the leaves of *Leucas aspera* and leaves of *Vitex negundo* showed good larval mortality against the larvae of *Culex quinquefasciatus* when compared to hexane, diethyl ether, dichloromethane and methanol extracts. The ethyl acetate extract of leaves of *Ocimum sanctum* produced significant mortality against *A. aegypti* and *C. quinquefasciatus*, with LC$_{50}$ values of 425.94 and 592.60 ppm (Anees, 2008). Bagavan et al. (2009) reported that the ethyl acetate extract from the leaves *Ocimum canum* and *Ocimum sanctum* showed good larvicidal activity against the larva of *Anopheles subpictus* (LC$_{50}$ = 88.15, 21.67 ppm and LC$_{90}$ = 528.70, 98.34 ppm respectively) and the ethyl acetate extracts of *O. sanctum* against the larvae of *Culex tritaeniorhynchus* (LC$_{50}$=109.12 ppm and LC$_{90}$ = 646.62 ppm). The ethyl acetate extract of leaves of *O. sanctum* also showed larvicidal potential against the nymph of *Aphis gossypii* (LC$_{50}$ = 73.27 ppm and LC$_{90}$ = 338 ppm).
Ali et al. (2013) reported that the ethanol and water mixture (3:1) extracts of Ulva lactuca, Sargassum microystum, Caulerpa scalpelliformis, C. racemosa, C. toxifolia, Gracilaria corticata, Turbinaria decurrens and T. conoides against mosquito larvicidal activity. Among the seaweed extracts, C. racemosa showed toxicity against 4th instar larva of A. aegypti, Culex quinquefasciatus and Anopheles stephensi with equivalent LC$_{50}$ values of 0.0556, 0.067 and 0.0661 µg/mL, respectively.

The methanol extract of Caulerpa scalpelliformis showed the larvicidal effect against Culex pippens with a reported LC$_{50}$ value of 338.9 ppm (Cetin et al., 2010). The methanol extract of Westiellopsis sp. (blue-green alga/cyanobacterium) active against fourth instar larva of Aedes aegypti with LC$_{50}$ value of 55.84 ppm (Rao et al., 1999).

In this study, the ethyl acetate extract of Stoechospermum marginatum showed the highest larvicidal activity. Marine brown algae are prolific producers of secondary metabolites, i.e. sesquiterpenoids, diterpenoids and compounds of mixed biosynthesis origin. Dictyotaceae are rich sources of bioactive terpenes that could be an evolutionary response of the brown algae to herbivore. The species of Dictyota and Padina were found to produce terpenoids such as diterpene and sesquiterpene (De Paula et al., 2011). This larvicidal effect of brown algae may be due to phenolic, terpenoids or unsaturated fatty acids
(Schnitzler et al., 2001). It was reported in the literature that the cytotoxic activity of some red and brown algal species could be attributed to the presence of a mixture of organic acids (Kamenarska et al., 2002). Many of the secondary metabolites produced by plants for its protection against microorganisms and predator insects are natural source for the discovery of new plant based products to combat Aedes aegypti. Many researchers have been focused on natural products for controlling Aedes mosquito as insecticide and larvicide with varied results (Chariandy et al., 1999).

5.6. LARVICIDAL ACTIVITY OF ISOLATED COMPOUNDS Sm2 AND Ul FROM SELECTED MARINE MACRO ALGAE

The fats and fatty acids from marine algae may play an important role in the formation of many other bioactive secondary metabolites since some fatty acids have been shown to possess antibacterial activity (Barbosa et al., 2007; Oh et al., 2008). Harada et al. (2000) reported that the unsaturated fatty acids (oleic acid, linoleic acid and linolenic acid) were reported to be more toxic than unsaturated fatty acids (myristic acid, palmitic acid and stearic acid) towards A. albopictus larva.

5.6.1. Larvicidal activity of 6-oxa-3-thiaoctanic acid (Sm2, a fatty acid)

In the present study, the 6-oxa-3-thiaoctanic acid isolated from the ethyl acetate extract of Stoechospermum marginatum had a remarkable larvicidal activity against A. aegypti. Maragathavalli et al. (2012) evaluated that the larvicidal activity of mosquito and phytochemical
screening of methanol extract from the leaves of *Azadirachta indica* showed the presence of chemical composition of caproic acid, 4-butoxy butanol, oleic acid, decanoic acid, 8 methyl, methyl ester, N-methyl N-N-di(2-(4-pyridyl)ethyl)-(2-pyridyl)ethylamine, 6(E),9(Z),13(E),pendedectriene, phytol, *cis*,*cis*,*cis*-7,10,13-hexadecatrienal. The fatty acid constituents, linoleic acid and oleic acid isolated from *Dirca palustris*, exhibited mosquitocidal activity against 4th *A. aegypti* larvae with LD$_{50}$ value of 100 µg/mL at 24 h (Ramsewak *et al.*, 2001).

In the present study, larvicidal activity the 6-oxa-3-thiaoctanic acid (*Sm2*) from *S. marginatum* showed a remarkable larvicidal activity against larva of *A. aegypti* (LC$_{50} = 42.9$ and LC$_{90} = 101.5$ ppm values at 24 h and LC$_{50}$ and LC$_{90}$ values of 22.9 and 59.1 ppm at 48 h, respectively). The fatty acid methyl ester extracts of *Vitex altissima*, *V. negundo* and *V. trifolia* were also reported to have larvicidal activity against 4th instar larvae of *Culex quinquefasciatus* (Kannathasan *et al.*, 2008).

5.6.2. Larvicidal activity of oleic acid (*Sm2*, a fatty acid)

Abdul Rahuman and Venkatesan (2008) found that oleic and linoleic acids were quite potent toxic against the 4th larvae of *A. aegypti, Anopheles stephensi* and *Culex quinquefasciatus*. Tare and Sharma (1991) reported that the larvicidal properties of different fatty acids constituents against *A. aegypti* and found that oleic acid was the most effective one.
In the present study, the oleic acid isolated from the ethyl acetate extract of *U. lactuca* had a remarkable larvicidal activity against *A. aegypti*. Rahuman *et al.* (2008) reported that the oleic and linoleic acids isolated from *Citrullus colocynthis* against *A. aegypti* with LC$_{50}$ value of 8.80, 18.20 ppm and LC$_{90}$ value of 35.39, 96.33 ppm (after 24 h), *Anopheles stephensi* with LC$_{50}$ value of 9.79, 11.49 ppm and LC$_{90}$ value of 37.42, 47.35 ppm (after 24 h) and *Culex quinquefasciatus* with LC$_{50}$ value of 7.66, 27.24 ppm and LC$_{90}$ value of 30.71, 70.38 ppm (after 24 h) respectively.

In the present study, larvicidal activity the Oleic acid (Ul) from *U. lactuca* showed a remarkable larvicidal activity against *A. aegypti* (LC$_{50}$ = 48.8 and LC$_{90}$ = 112.6 ppm values at 24 h and LC$_{50}$ and LC$_{90}$ values of 31.3 and 80.1 ppm at 48 h respectively). Kannathasan *et al.* (2011) reported that the methyl-$p$-hydroxybenzoate isolated from *Vitex trifolia*, showed 100 % larval mortality at 20 ppm against *C. quinquefasciatus* and *A. aegypti* with LC$_{50}$ values of 5.77 and 4.74 ppm, respectively. Joseph *et al.* (2004) reported that neotenone, as isoflavonoids isolated from *Nerorautautaenia mitis* tubers resulted 100 per cent mortality of *Anopheles gambia* larvae at 20 ppm. Cantillo-Ciau *et al.* (2010) studied the antiprotozoal activity of brown alga, *Lobophora variegata* against *Giardia intestinalis, Entamoeba histolytica* and *Trichomonas vaginalis*. They have extracted antiprotozoal compound, the major compounds included
-O-palmitoyl-2-O-myristoyl-3-O-(6''-sulfo-aD-quinovopyranosyl)-glycerol; 1,2-di-O-palmitoyl-3-O-(6''-sulfoa-D-quinovopyranosyl)-glycerol and a new compound identified as 1-O-palmitoyl-2-O-oleoyl-3-O-(6''-sulfo-a-Dquinovopyranosyl)-glycerol.

**5.7. MOLECULAR MODELING OF THE ISOLATED LARVICIDAL COMPOUNDS, Sm2 AND Ul**

In the present study, computational techniques have enabled researchers to estimate the binding affinity of compound and evaluation in lab. Molecular docking used to find out the binding orientation of the small molecules against their targets. Thus, molecular docking is considered as an important technique in drug designing and screening of novel compounds against this dreadful and challenging diseases (Lengauer and Rarey, 1996). The current study focused on the docking of the compound against NS3 Protease-helicase form dengue virus protein.

Huang and Zou (2007) assessed the molecular docking which is a widely-used computational tool for the study of molecular recognition, which aims to predict the binding mode and binding affinity of a complex formed by two or more constituent molecules with known structures. An important type of molecular docking is protein-ligand docking because of its therapeutic applications in modern structure-based drug design.
In the present study, molecular modeling process is divided into the four steps *viz.* preparing of protein, preparing of ligand, generating of the receptor grid and docking the ligand and molecular docking runs of all known co-crystallized bound compounds (*Sm*2 and *Ul*) to (2WHX) NS3 protease-helicase targeted proteins. In that, the compound pose had the good glide score and energy compared to other poses for 2WHX (the best glide score of $\text{Sm}2 = -7.1$ and $\text{Ul} = -7.2$ kcal mol$^{-1}$) and also confirmed the result with good binding energy. Dengue virus has four serotypes (Khan *et al.*, 2008a) but any inhibitor against the binding pocket of NS2/NS3 protease could work against all the serotypes (Li *et al.*, 2005). Like other flaviviruses dengue virus, NS3 protease has been declared as significant drug target. Catalytic triad is important in viral replication therefore, any disruption in it may block the replication of virus (Van Hell *et al.*, 2009). Mangiferin and gallic acid are the main components of *Mangifera indica*, have good docking studies on almost all the viruses. Mangiferin is reported to antagonize cytopathic effects of HIV have good docking studies against HIV protease. The same compound was reported to have potential activity against HIV protease (Wang *et al.*, 2011). In another study, gallic acid which gave excellent docking scores on HIV reverse transcriptase and found to have inhibitory activity on the same (Nutan Modi *et al.*, 2013). DF and DHF are caused by the dengue virus, which is a member of the Flaviviridae. There are four serotypes of dengue virus, DEN1, DEN2,
DEN3 and DEN4 with DEN2 being the most prevalent. The RNA genome of DEN2 contains 10723 nucleotides and encodes a large polyprotein precursor of 3391 amino acid residues which consist of three structural proteins (C, prM and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Irie et al., 1989). Optimal activity of the NS3 serine protease is required for the maturation of the virus and the presence of the NS2B co-factor is a pre-requisite for the optimal catalytic activity of NS3 (Bianchi and Pessi, 2002).

5.8. CYTOTOXICITY OF THE ISOLATED COMPOUNDS ON VERO CELL LINE

In the present study, cytotoxicity effect of the six isolated active compounds against African green monkey kidney cell line (Vero). Many plant extracts and isolated compounds have been tested in vitro for cytotoxicity by using different human cell lines (prostate, stomach, liver colon etc.) as well as animal cells such as monkey kidney cells (Jo et al., 2005). Cell culture toxicity testing is a valuable and inexpensive approach for short term testing. A test should be able to provide information on the dose-effect relationship including the dose range for potential exposure and risks to humans. Cytotoxicity of plant extracts and isolated compounds should be evaluated before their impact in drug discovery is taken into consideration (Lall and Meyer, 2000). Cao et al. (2006) reported the isolation of triterpene saponins which showed significant cytotoxicity activity against various cell lines.
A key milestone that lead molecules have to reach for further development as drug candidates, is that they have to be declared ‘safe’ for use. In such a way, in vitro cytotoxicity assays are useful to define basal cytotoxicity, for example the intrinsic ability of compounds to cause cell death as a result of damage to several cellular functions (Bouaziz et al., 2006). Cytotoxicity assays are also necessary to define the concentration range for further and more detailed in vitro testing to provide meaningful information on parameters such as genotoxicity or programmed cell death (Eisenbrand et al., 2002). A number of assays and various cell types have been used with different responses to study the cytotoxicity and none can be considered as standard. Vero cells have been used with some frequency with the advantage of easy availability and fast growth (Bouaziz et al., 2006).

In the present study, the isolated six compounds viz., Sm1, Sm2, Ca1, Ca2, Ge and Ul were studied for its cytotoxic potential on Vero cells lines for their safe use. MTT assay indicated that Sm1, Ca1 and Ca2 had the IC₅₀ value of >100 μg/mL. However Sm1, showed the little toxicity to the Vero cells with the IC₅₀ value of 39.15 μg/mL. In the present investigation, dose response effects of the isolated compounds were clearly observed, since Vero cell viability gradually decreased with the increase of compounds concentrations. The results of the MIC values showed that the isolated compounds inhibited all the bacterial species tested at a maximum concentration of 12.5 μg/mL,
which is relatively safe to Vero cells. Moreover, the criteria of cytotoxic activity, as established by the American National Cancer Institute, for future studies, the IC$_{50}$ value should be $>30 \ \mu g/mL$ (Suffness and Pezzuto, 1990). According to this measure, the isolated compounds were non toxic on Vero cells.

The morphological changes in the cells were observed more prominent in treated cells showing extensive blebbing and vacuolation suggest autophagic mechanism of cell death (Vijayarathna and Sasidharan, 2012). The distinct effects of these extracts may be due either to the phytodiversity or diverse mechanisms associated with each of the phytocompounds. These data suggest that the non-toxic effect of the compounds thus making it suitable for the preparation of drugs involved in the treatment of various diseases. In addition, it revealed that the cytotoxic activity was not related to lytic properties or membrane instability induced by the compounds (Costa-Lotufo et al., 2005).

Thus, the present study strongly literates the medicinal importance of the marine macro algae and scientifically validate it for use as a component of medicinal preparations.