Chapter 5

Discussion

Marine algae/seaweeds are gaining increasing interest due to their novel source of compounds with potent medicinal properties. Earlier, seaweeds are used by various practitioners based on the traditional knowledge but due to the lack of scientific evidence this did not gain much importance in drug discovery (Layer et al., 1986). Recently, phytocompounds present in the seaweeds are reported for antidiabetic property by inhibiting certain class of enzymes (α-amylase, α-glucosidase and DPP-IV) (Liu et al., 2012). It is anticipated that the isolation, characterization and pharmacological study of unexplored marine algae can be useful in the discovery of novel antidiabetic compounds with high biomedical value. Therefore, in this study we focus the antidiabetic activity of seaweeds/marine algae collected from southern coast of India which has not been explored previously.

In this study, seaweeds were collected mainly from the coastal regions of Kerala (Azheekal and Vizhinjam) and Tamilnadu (Mandapam, Cuddalore, Kovalam, Muttom and Kadiapattanam). According to Yadav et al., 2015 and Muthukrishnan et al., 2013, more than one third of the seaweeds with richest diversity makes southern coast of India as an integral part of seaweed resources. The phytocompounds present in the seaweeds will depend on various factors (species, temperature, geographical location, salinity, season and stress tolerance) (Norziah and Ching, 2002; Wong and Cheung, 2000). Therefore, seaweeds were extracted serially from nonpolar–polar and preliminary phytochemical analysis was carried out check the presence of major phytocompounds. The class of compounds present in the extracts was reported for various biological activities like antiviral, anti-inflammatory, antidiabetic, antioxidant and anticoagulant activity (Cumashi et al., 2007; Lee and Jeon, 2013; Ghosh et al., 2009). Among them, phenolic compounds and flavanoids (naringin) are well documented for its antioxidant, hypocholesterolemic, antiatherogenic, anti-inflammatory and antidiabetic activity (Lordan et al., 2013; Jung et al., 2004; Choi et al., 2001; Jain and Parmar, 2011). Recently, alkaloids isolated from the root of Aerva lanata Juss (Amaranthaceae) have also reported for its antidiabetic activity (Agrawal
et al., 2013). The synergetic action of these phytocompounds and/or individual phytocompounds might be responsible for their respective bioactivity.

The ability of seaweed extracts to inhibit α-amylase and α-glucosidase is found to be an efficient strategy for the control of DM (Unnikrishnan et al., 2014). Therefore, to understand the inhibitory effects they were compared with a standard acarbose. Among the various extracts tested, methanolic extract of *Turbinaria ornata* (96%), *Chaetomorpha aerea* (85.89%), *Ulva reticulata* (61%), *Chaetomorpha antennina* (53%), *Chlorodesmis* (71%), *Gracilaria, corticata* (53%), ethyl acetate extract of *Sargassum polycystum* (76%), *Porteria hornemanni* (50%) and petroleum ether extract of *Sargassum wightii* (62%) showed highest inhibition against α-amylase at a concentration of 1000 μg/ml. The obtained IC₅₀ value for methanolic extract of *Turbinaria ornata* which showed highest inhibition against α-amylase was found to be 250.9 μg/ml. Similarly, methanolic extract of *Ulva reticulata* (97%), *Chaetomorpha antennina* (97%) and *Sargassum polycystum* (95%), ethyl acetate extract of *Sargassum wightii* (91%), petroleum ether extract of *Gracilaria, corticata* (88%), *Ulva linza* (86%), *Ulva lactuca* (82%) and acetone extract of *Turbinaria ornata* (83%), *Porteria hornemanni* (81%) also showed significant inhibition against α-glucosidase. The observed IC₅₀ value for *Ulva reticulata* (147.2 μg/ml), *Chaetomorpha antennina* (121.3 μg/ml) and *Sargassum polycystum* (289.7) were similar to standard acarbose. Both α-amylase and α-glucosidase can be compared with a standard acarbose (171.8 and 196.8 μg/ml) (Santeusanio and Compagnucci, 1994).

The above mentioned results showed a concentration (250-1000 μg/ml) dependent inhibition against α-amylase and α-glucosidase. Seaweeds belong to the phylum Phaeophyta (*Turbinaria ornata, Sargassum polycystum, and Sargassum wightii*), Rhodophyta (*Porteria hornemanni, Gracilaria, corticata*) and Chlorophyta (*Ulva reticulata, Chaetomorpha antennina, Chaetomorpha aerea, Chlorodesmis, Ulva linza, Ulva lactuca*) posses’ notable action against α-amylase and α-glucosidase. Previously, seaweeds belongs to the phylum Phaeophyta (*Sargassum polycystum, Ecklonia stolonifera, Eisenia bicyclis, Turbinaria ornata*) showed similar antidiabetic and antioxidant activity (Maeda et al., 2007; Vaugelade et al., 2000; Taskinen, 2002; Moon et al., 2011; Ananthi et al., 2010). Among them, *Sargassum polycystum* which is rich in soluble fibres and sulphated polysaccharide have reported for reducing
cholesterol, antihyperglycemia, dyslipidaemia, intestinal glucose absorption and increased insulin sensitivity in diabetic models (Maeda et al., 2007; Vaugelade et al., 2000; Taskinen, 2002; Motshakeri et al., 2013). Apart from these our study validates the antidiabetic activity of Sargassum (S. polycystum and S. wightii) species and Turbinaria in both in vitro and in vivo models.

In the same way, marine red algae (Rhodophyta) also been studied for its bioactivity over the past two decades for its natural products chemistry (Fuller et al., 1992; Kim et al., 2008). Already, bromophenols present in red seaweed, Grateloupia elliptica shown intestinal α-glucosidase inhibition (Kim et al., 2008), while extracts of Hypnea musciformis shown antihyperglycemic and antioxidant activity in diabetic animals (Anandakumar et al., 2008). In this study, five red seaweeds (Porteria hornemannii, Gracilaria corticata, Gracilariopsis lemaneiformis, Aspirogobsis taxiformis, Gelidiopsis) were screened for in vitro antidiabetic activity. Among them two seaweeds (Porteria hornemannii, Gracilaria corticata) showed significant enzyme inhibitory activity. It is the first report on the potential of P. hornemannii and Gracilaria corticata extracts on the inhibition of key metabolic enzymes linked to diabetes. Seaweeds belongs to the phylum Chlorophyta (Chaetomorpha aerea, Enteromorpha intestinalis, Chlorodesmis, Cladophora rupestris, Chaetomorpha antennina, Ulva reticulata, Ulva linza and Ulva lactuca) also showed significant antidiabetic activity by inhibiting the enzymes α-amylase and α-glucosidase. Among them Ulva reticulata and Chaetomorpha antennina showed highest inhibition against α-amylase and α-glucosidase. Previous reports shows that Ulva rigida ethanol extract and Ulva linza exhibit antihyperglycemic, antigenotoxic, anti-oxidative and antihypertensive activity in vivo (Celikler et al., 2009; Ramirez-Higuera et al., 2014). Three seaweeds (C. aerea, E. Intestinalis and C. rupestris) were previously reported for its antibacterial and antioxidant activity (Akköz et al., 2011; Pierre et al., 2011). But they are not screened for its α-amylase and α-glucosidase inhibitory activity to elucidate the hypoglycaemic activity in vitro. Further, seaweeds (Ulva reticulata and Chaetomorpha antennina) showed highest α-amylase and α-glucosidase inhibition will be advised for the treatment of DM, and also considered as a candidate for further studies to isolate carbohydrate hydrolyzing enzyme inhibitors.
Apart from α-amylase and α-glucosidase, we have also investigated the effect of various extracts against DPP-IV. Among the various seaweed treated methanolic extract of *Sargassum polycystum* (58%), *Sargassum wightii* (57%), *Turbinaria ornata* (55%), *Gracilaria corticata* (46%), *Gracilaripsis lemameiformis* (45%), *Chaetomorpha antennina* (45%), *Ulva reticulata* (44%) and ethyl acetate extract of *Porteria homemannii* (56%) showed significant inhibition against DPP-IV which is compared with a standard Diprotin A (65%). Earlier, natural DPP-IV inhibitors (berberine and naringin) were isolated from plants and fruits (orange peel) to investigate its hypoglycaemic action *in vitro*, *in vivo*, and *in silico* models to know the possible mechanisms (Al-Masri et al., 2009; Parmar et al., 2012). The *in vitro* DPP-IV inhibitory activity of berberine (IC$_{50}$-13.3 mM) and naringin (68.76%) showed similar inhibitory pattern as our study (Al-Masri et al., 2009; Parmar et al., 2012). Therefore, our study which showed significant DPP-IV inhibitory activity can be studied later for its detailed mechanism through various cell line and animal models to quantify the amount of insulin released/min under *in vitro* and *in vivo* conditions.

Furthermore, the extracts were also screened for *in vitro* antioxidant activity by DPPH method. The major advantage of using DPPH is that the reduced free radical can be measured by using spectrophotometric method. The present study aims to give a conclusive data about the antioxidant ability of the tested extracts. The results presented here shows that the seaweeds belong to the phylum chlorophyta (*Cladophora rupestris*, *Ulva lactuca*, *Ulva reticulata*, *Ulva linza*) and phaeophyta (*Turbinaria ornata*, *Sargassum wightii*) shows highest scavenging activity. As previously reported by (Qi et al., 2005) seaweeds belong to the phylum chlorophyta (*Ulva pertusa*) exhibited strong antioxidant activity including superoxide, hydroxyl scavenging, reducing power and chelating ability. According to (Celikler et al., 2009), *Ulva* species are rich in polyphenols and the antioxidant property of polyphenols are well documented by (Karawita et al., 2005). Brown seaweed (*Sargassum wightii*) was reported for excellent free radical scavenging activity due to the presence of various phytochemicals (flavanoids, terpenoids and phenolic compounds) (Matanjun et al., 2008). *Sargassum* species rich in polysaccharides (alginites, fucoidan and laminaran) have reported for antioxidant activity which prevents the destruction of pancreatic β-cell that leads to diabetes (Riou et al., 2010). As previously reported by
(Chattopadhyay et al., 2010; Ananthi et al., 2010), fucoidan fractions isolated from *Turbinaria conoides* and aqueous extract of *T. ornata* shows similar scavenging activity on DPPH radicals. Our results were similar to these reports which confirm the antioxidant activity of *T. ornata*. Similarly, marine red seaweeds/Rhodophyta (*Porteria hornemannii*, *Gracilaria*, *corticata*, *Gracilariopsis lemaneiformis*) exhibit moderate to less antioxidant activity. Earlier reports shows that two seaweeds (*Porteria hornemannii*, *Sphaerococcus coronopifolius*) belong to the phylum Rhodophyta showed less free radical scavenging activity 11.84 and 19.54% respectively (Senthilkumar et al., 2013; Paiva et al., 2012). As compared to previous studies our results supports the antioxidant activity of seaweeds belongs to the phylum rhodophyta.

The chemical components present in the seaweed extracts which showed significant antidiabetic activity *in vitro* were analyzed by GC-MS. Among them, methanolic, ethyl acetate and petroleum ether extracts of *Sargassum polycystum*, *Sargassum wightii*, *Chaetomorpha antennina* and *Gracilaria corticata* showed the presence of fucosterol, an antidiabetic compound previously isolated from marine algae (*Pelvetia siliquosa*, *Eisenia bicyclis*, and *Ecklonia stolonifera*) (Lee et al., 2004; Jung et al., 2013). It is previously reported for its ability to inhibit rat lens aldose reductase (RLAR), human recombinant aldose reductase (HRAR), protein tyrosine phosphatase 1B (PTP1B) and α-glucosidase (Jung et al., 2013). Further, its kinetic study reveals that fucosterol showed mixed type inhibition against RLAR and HRAR, it also inhibits PTP1B noncompetitively (Jung et al., 2013). Hence, it can be considered as a promising source for the management of diabetic complications (Jung et al., 2013). We also identified the presence of fucosterol in *G. corticata* belongs to the phylum Rhodophyta for the first time. Methanolic and ethyl acetate extract of *Ulva reticulata*, *Chaetomorpha antennina* and *Porteria hornemannii* showed the presence of ascorbic acid (vitamin C) is a known antioxidant which was isolated from several seaweeds and plants (Celikler et al., 2009). Thus, the presence of ascorbic acid in the extracts play a major role in the prevention and/or betterment of pathological damage caused by hyperglycemia-induced oxidative stress associated with diabetes (Lee et al., 2010). Ascorbic acid, esters of fumaric acid and oleic acid are present in ethyl acetate extract of *Sargassum wightii*. These compounds are well documented for
various treatments like wound healing, huntingtons disease, neurodegenerative diseases and for the prevention of atherosclerosis (Okwu and Ighodaro, 2010; Ellrichmann et al., 2011; Parthasarathy et al., 1990). Acetone extract of T. ornata revealed the presence of hentriacontane, a natural compound present in Oldenlandia diffusa for the treatment of cancer in Asia (Kim et al., 2011) and also have reported activities like anti-inflammatory and antioxidant properties (Kim et al., 2011; Agoramouthy et al., 2007). Currently there is no information regarding the antidiabetic effects of hentriacontane. Previous reports states that most of the identified compounds (tetradecanoic acid, 10, 13-dimethyl-methyl ester, n-hexadecanoic acid, l-(+)-ascorbic acid 2, 6-dihexadecanoate, 6-octadecenoic acid, phenol 2,4-bis (1,1-dimethylethyl)) have been reported for various biological activities like antioxidant, anti-inflammatory, antibacterial and antifungal activity (Huang and Wang, 2004; Aparna et al., 2012; Agoramooorthy et al., 2007).

Certain metabolites found in the seaweed extracts can be toxic, so it is necessary to evaluate the extracts prior to animal study. Hence, the cytotoxicity of seaweed extracts using mouse macrophage cells (J774) by MTT assay. The assay showed no observed toxicity in lower concentrations. Similarly, the tested extracts did not showed any DNA fragmentation and/or lysis of human erythrocytes even at higher concentrations (1000 μg/ml). Overall the extracts found to be less toxic in studied models.

The chromosomal aberration (CA) assay evaluates the different types of induced aberrations seen in the human leukocytes (Cavalcante et al., 2014). CA testing of seaweed extracts will reveal the unidentified and unregulated toxicants present in it. Our results demonstrate that, except G. corticata all other seaweeds (S. polycystum, U. reticulata and C. antennina) don’t possess any genotoxic effect on human leukocytes. Both, petroleum ether and methanolic extracts of G. corticata are moderately toxic to the chromosomes under in vitro conditions. The extracts exhibit dose-dependent chromosomal toxicity. Whereas, S. polycystum, U. reticulata and C. antennina extracts (250-1000 μg/ml) display insignificant chromosomal changes when compared with negative control. Various aberrations seen in the cells include chromosomal break, chromosome gap, chromatid gap, pulverization, and dicentric chromosome. Leukocytes treated with G. corticata extracts showed chromosome
breaks, whereas cells treated with *C. antennina* showed breaks along with dicentric chromosomes in some metaphase cells. The frequency of chromosomal aberration is less in case of *S. polycystum, U. reticulata* and *C. antennina* treated cells compared to *Gracilaria corticata*. Earlier studies showed that the seaweeds generally do not exhibit mutagenicity and genotoxicity (Silva et al., 2007).

Among the various *in vitro* toxicological tests, plasmid-nicking assay has been widely approved as a valid indicator of genotoxicity (Siddiqui, 2011). Therefore, circular plasmid DNA (pUC18) was selected for testing the toxic effects of extracts in DNA. Hydrogen peroxide (30%), a known mutagenic agent was used as a positive control. The results indicate that among the various extracts, petroleum ether and methanolic extracts of *G. corticata* caused fragmentation of DNA at all the concentrations (250-1000 μg/ml). Whereas, methanolic extracts of *S. polycystum, U. reticulata* and *C. antennina* did not show any fragmentation of DNA even at a higher concentration (1000 μg/ml).

In this study, seaweeds (MEUR, MESP, MECA and AETO) showed highest antidiabetic activity *in vitro* was further investigated for anti hyperglycaemic activity in STZ induced diabetic rats. STZ (glucosamine – nitrosourea compound) is a chemotherapeutic agent which selectively destruct pancreatic islet (β) cell through the release of nitric oxide and also generates SOD anions which interacts with mitochondria that leads to diabetes and its associated complications (Papaccio et al., 2000; Szkudelski, 2001). The intraperitoneal induction of STZ (45 mg/kg body weight) leads to insulin resistance and hyperglycemia (˃ 350 mg/dl). Diabetic rats treated with MEUR (46.21%), MESP (40.73%), MECA (40.13%) and glibenclamide (73.56%) showed maximum reduction in FBG level when compared to AETO (23.41%). The hypoglycaemic effects of extracts are due to insulin sensitivity for glucose uptake in target tissues rather than insulin secretion. These extracts also help to regulate the carbohydrate digestion rate and/or absorption by inhibiting α-amylase and α-glucosidase. MEUR, MESP, MECA and glibenclamide treated rats also improved their body weight due to its better control over hyperglycaemic state and increased synthesis of structural proteins in the diabetic rats (Eliza et al., 2009). The level of serum lipids (hypercholesterolemia and hypertriglyceridemia) usually elevates in STZ induced diabetic rats. Test and glibenclamide treated rats significantly
reduces the level of triglycerides and total cholesterol when compared to the untreated and this elevates hypercholesterolemia and reduce the risk of atherosclerosis (Taskinen, 2002). Several seaweeds including S. polycystum and Ulva rigida have shown to posses’ anti-hyperglycaemic, anti-genotoxic and hypocholesterolaemic effect in mammals and thereby reduce the risk of cardiovascular diseases (Mohamed et al., 2012; Motshakeri et al., 2013; Celikler et al., 2009). Treated extracts and standard group also showed a reduction in the level of ALT and AST. This marked the hepatoprotective effect of seaweed extracts. Other parameters like total protein, urea and albumin were found quite similar to normal rats. Histological studies of kidney liver and pancreas revealed that the extracts were non toxic. Finally, MEUR (46.21%) and MESP (40.73%) which showed highest antihyperglycemic activity were selected for bioactivity guided isolation of active compounds responsible for its antidiabetic action.

In our study various factions were isolated from MESP, among them F6 (methanol) fraction showed highest antidiabetic activity in vitro followed by F4 (chloroform) and F5 (ethyl acetate) fraction. Matanjun et al., 2008, reported that alcoholic extract of S. polycystum are rich in phenol and flavanoid content with excellent free radical scavenging activity. In our study F4 fraction (87.13 μg/ml) showed highest phenolic content followed by F5 (38.98 μg/ml) and F6 (23.96 μg/ml). Phenolic compounds present in the seaweeds are excellent antidiabetic agents (Mohamed et al., 2012). GC-MS results also validate the presence of phenol content present in the F4 fraction of S. polycystum. Fucosterol is another phytosterol present in various brown seaweeds (Pelvetia siliquosa, S. polyceratium) with various pharmacological properties (antidiabetic, antioxidant and adntioxidant) (Lee et al., 2003; Lee et al., 2004). Our results also support the antidiabetic activity of fucosterol in STZ induced diabetic rats (Lee et al., 2004), due to the presence of fucosterol and Z,Z-6,28-heptatriacactontadien-2-one in F5 fraction of MESP. Several plants are reported for the antidiabetic property of fatty acids (Orhan et al., 2012). In this study, 9-octadecenoic acid (z)-hexadecyl ester a fatty acid isolated from F6 fraction of S. polycystum showed highest inhibition against α-amylase and α-glucosidase in vitro. Further in silico studies were done to find out the binding efficiency of 9-octadecenoic acid (z)-, hexadecyl ester against α-amylase and α-glucosidase.
9-octadecenoic acid (z) - hexadecyl ester which showed highest activity in the active fraction (F6) of MESP were subjected for docking and MD simulation studies for better understanding the ligand-protein interaction in detail. Therefore, 9-octadecenoic acid (z) - hexadecyl ester and the same compound without derivative (9 octadecenoic acid) were docked into the active site of α-glucosidase and α-amylase. The compound 9-octadecenoic acid (z)-hexadecyl ester formed strong hydrophobic bonds with catalytic (ASP215, GLU277, ASP352) and active site (ASP69, ARG442, ARG446, HIS351, TYR158, HIS280, ARG315) residues of α- glucosidase. Similarly the compound also interacts with the catalytic (ASP197, GLU 233, ASP300) and active site (HIS101, TRP58, TRP59, TYR62) residues of enzyme α-amylase. Other important active site residues (HIS101, TRP58, TRP59, and TYR62) and hydrophobic interactions are also a crucial element for binding the compound with target protein (Ghosh et al., 2014; Qian et al., 1994). Molecular dynamic (MD) stimulations are also carried out to analyze the overall stability of enzyme, enzyme-ligand complexes on nano second timescale. In this study we have analyzed the MD stimulation of 9-octadecenoic acid (z)-hexadecyl ester with α-glucosidase and α-amylase in 10 ns time scale. 9-octadecenoic acid (z)-hexadecyl ester shows strong interactions with catalytic residues (GLU277 and ASP352), polar residues of binding pocket (ARG213 and HIS351), hydrophobic residues of active site (PHE301, PHE303) glucose binding region (TYR158, HIS280, THR310, PRO312, LEU313, ARG315) and also various other residues of α-glucosidase (GLN279, GLN353, ASP307, ASN350, THR306, ASP242, ARG359) throughout the stimulation. Similarly, our compound is interacting with a long loop (ALA345, ASN347, VAL354, ASN355, and TRP357) and moving loop (HIS305) region of α-amylase which can adopt different conformation of ligand binding site that is strongly associated with enzyme function. Certain interacting residues (GLU63, TRP59, TRP357, and PRO54) are also retained in 10 ns. Therefore, our findings suggest that 9-octadecenoic acid (z) - hexadecyl ester can have a potent inhibition against α-glucosidase and α-amylase.

Since, F4 fraction of MEUR was found to posses’ highest antidiabetic activity in vitro were subjected to various chromatographic and spectroscopic methods. Five compounds namely, nonane, hexadecanoic acid, 1-dodecanol, Cyclodecane methyl and phenol, phenol 3,5-Bis(1,1-Dimethylethyl) were identified in the F4 fraction of
MEUR. Further, *in vitro* enzyme inhibitory, insulin secretion studies in primary islet cells, molecular docking and dynamic simulation studies clearly validate the synergistic action of five compounds present in the active fraction (F4). Due to the presence of fatty acids, fatty alcohols and phenols, F4 fraction showed highest antidiabetic activity *in vitro*. The fatty acids (palmitic and/or hexadecanoic acid) isolated has reported for the insulin release from the beta cells as well as enhanced glucose uptake in skeletal muscles (Orhan et al., 2012; Pu et al., 2011). The presence of phenols and phenolic derived compounds confers its antidiabetic action (Mohamed et al., 2012). Due to the presence of all these bioactive components, active fractions (F4) shows highest antidiabetic activity *in vitro* and further this fraction (F4) were subjected to *in vivo* antidiabetic studies in three general models. Among the three models, normoglycaemic used to evaluate the hypoglycaemic effect, glucose loaded rats for the intestinal absorption and STZ-loaded rats for the antihyperglycaemic effect (Orhan et al., 2012). Active fraction (F4), rich in fatty acids and phenols posses’ antidiabetic activity by increasing the insulin release from beta cells and also by delaying the carbohydrate digestion rate. Therefore, active fraction (F4) can be used for amelioration of diabetes and its complications significantly.