

1. INTRODUCTION

Natural products are prolific sources of potential phytochemicals for novel drug development [1], [2]. Nearly 30% of the leading drugs in the market are obtained from plants [3]. Moreover, 50% of the existing drugs are based on natural products or contain lead molecules from natural origin [4]. The toxicity induced by the synthetic compounds in clinical trials has turned the attention of the drug industry towards the use of natural products. Together, the refinement of analytical techniques has increased the utilization of natural products in drug discovery and development.

The ocean is a complex environment, encompassing 75% of living organisms from 36 phyla [5], [6]. As taxonomic diversity is related to chemical diversity, the marine natural products (MNPs) are entirely different in their specificity and structure. These MNPs enable the marine organisms to survive in the harsh marine environment. Hence, the MNPs could be potential candidates for the discovery of novel drugs and lead molecules. Based on this concept, the utilization of marine organisms has widened in the recent years which has caused the isolation of 28,000 MNPs [7 – 9].

Among the marine organisms, the marine algae are well documented. They are rich sources of phytochemicals with varied therapeutic potential. These include the antibacterial, antiviral, antioxidant, antidiabetic, antipyretic, anticancer, antimycobacterial, analgesic, anticoagulant, anti-inflammatory, antiprotozoan properties [7], [10 – 14].

Marine algae are eukaryotic, photosynthetic plants varying from the microalgae to the macroalgae [15], [16]. The microalgae are the unicellular phytoplanktons which are the major food producers in the ocean [17]. They dwell in the benthic and littoral zones of the sea and includes the diatoms, dinoflagellates and the blue green algae comprising 40,000 to 70,000 species [18]. The marine macroalgae (seaweeds) are multicellular and are classified into brown, green and red algae [19]. These macroalgae are found in the littoral zones of the sea [20].

Most marine algae are edible and form a part of the traditional food in China and Asia since 600 BC [21]. The timeline of MNPs entering the pharma market started with the commercialization of Kainic acid in 1900 for use as anthelmintic and as insecticide [22] followed by spongothymidine and spongouridine in 1950 [23]. Recently (till 2011), eight drugs of marine origin were approved by the Food and drug Agency (FDA) and European Union to cure various diseases. These include Cytrabine (1969), Vidrabine (1976), Ziconitide (2004), Omega 3 fatty acid ethyl ester, Trabectidin (2007), Eribulin Mesylate (2010), Brentunimab vedotin (2011) and Iota carrageenan [24].

The red algae grow in the intertidal and sub tidal zones of the sea. Nearly 10,000 species of red algae are reported globally. They are rich in phycoerythrin and phycocyanin which impart red colour to them. Red algae are abundant sources of bioactive compounds and widely used in therapeutics. These include antitumour [25],[26], antiviral [27 – 29], anthelmintic [30],[31], anti-inflammatory [32],[33], antioxidant [34],[35], antibacterial [36] and antimalarial [37],[38] activities.

In the current study, the red marine algae *Gelidiella acerosa* was utilized to test its antioxidant, anticancer, antimetastatic and anti-inflammatory potential. *Gelidiella acerosa* (Forsskal) belongs to the, Family *Gelidiellaceae*. The algae grows abundantly along the coastal regions of South India. It occupies the intertidal zone and grows by attaching to the rocks.

The algae is commercially utilized for the isolation of superior quality agar worldwide [39]. It is a source of various bioactives which can be used as nutraceuticals and in therapeutics [40]. *Gelidiella acerosa* was reported as a source of antioxidant [41], antibacterial [42], anticancer [43], contraceptive [44] activities. Further, the algae also was reported to inhibit acetyl choline esterase and butyl choline esterase enzyme activities [45]. The silver nanoparticles of the algae exhibited antifungal activity [46].

Although, previous studies have reported the therapeutic potential of the algae, the active compounds and their mechanism of action in cancer are still in their infancy. Hence, the current study was aimed in isolating and characterizing the bioactives

from *G.acerosa* and to investigate its antioxidant, anticancer, antimetastatic and anti-inflammatory activities in cancer.

Even though, seaweeds were utilized as a source of food and medicine since ancient times, the documentation of their therapeutic effects is very scarce. This may be due to the lack of sophisticated techniques needed for their isolation and characterization. But the recent advancements in the fields of analytical techniques, spectrophotometer and NMR techniques has speeded up the isolation and identification of MNPs [47]. In the recent decades, 28,000 MNPs have been reported. In the current study, the algae was extracted and the extracts were characterized by FTIR, HPLC, GC - MS and NMR techniques.

Cancer is a major health issue associated with high morbidity and mortality [48]. Based on the cancer statistics of WHO, the disease took a death toll of 8.8 million in 2015 and is expected to reach 22 million in 2030 [49]. Cancer can occur in all types of body cells and the cancers of the lung account for the major cancer-related deaths (1.69 million) [50]. The two major histological classification of lung cancer include NSCLC and SCLC. Among this, NSCLC contributes to 85% of lung cancer cases. Further, NSCLC comprises three subclasses, including small cell carcinoma, large cell carcinoma and adenocarcinoma. The lung adenocarcinoma accounts for 40% of NSCLC cases [51].

Based on severity and survival, lung carcinoma is stated as the lethal malignancy with only 18% patients surviving post – diagnosis [52]. Out of the 2 million cases reported in 2015, 1.7 million died in the same year [53]. The yearly incidence of lung cancer is reported to increase by 2% globally [54].

NSCLS is presented by symptoms including cough, hemoptysis (primary tumour), blockage in superior vena cava (intrathoracic spread) and bone pain (distant metastasis). Together weight loss, dyspnea, loss of appetite, fatigue, digital clubbing are also reported [55], [56]. The usual diagnostic procedures include tissue evaluation, staging and functional evaluation and computed tomography [57]. The treatment strategies employed depends on the stage and commonly involves radiation therapy, chemotherapy and surgery [58], [59].

The economic impact caused by cancer is increasing and the annual economic cost was reported as 1.16 trillion US\$ in 2010 [60]. Lung cancer is reported as the third most expensive cancer accounting for \$ 5647 USAD per patient [61].

Cancer is a group of disease [62] characterized by increased metabolism, abnormal functioning of mitochondria, peroxisomes, aberrant cell signaling and prolonged inflammation results in the excess production of Reactive oxygen species (ROS) [63]. ROS are removed by the cellular antioxidant enzymes especially the SOD, POX and CAT and by non – enzymes, including the flavonoids, vitamins and glutathione [64].

Superoxide dismutase (SOD) are metallo enzymes, ubiquitously expressed in cells, remove the superoxide and thus protecting the cells from oxidative stress [65], [66]. It is well established that increased metabolism, abnormal functioning of mitochondria, peroxisomes, cyclooxygenase, lipoxygenase, aberrant cell signaling and prolonged inflammation can result in the overproduction of ROS in carcinogenesis [67].

Antioxidants act by removing the free radicals and by enhancing the activity of antioxidant enzymes [68]. But the synthetic antioxidants (Beta hydroxyl toluene) induced toxicity in animals and hence a new source of natural antioxidant which is safe in the animal system is sought [69]. Based on this, and as *G.acerosa* was reported for its antioxidant activity, the current study analyzed the efficacy of *G.acerosa* in removing free radicles. The study also determined the efficiency of *G.acerosa* and its compounds to enhance the activity of SOD and POX under *in vitro* conditions.

Cancer results, when cells undergo multiple genetic changes and evolve mechanisms to evade apoptosis or programmed cell death. This results in the loss of homeostasis [70] resulting in uncontrolled cell division. The major obstacle in the treatment of cancer results due to evasion of apoptosis and tendency of cancer cells to spread and establish at new sites termed as metastasis.

Apoptosis is regulated either by the intrinsic or extrinsic pathways. Both these pathways activate caspase 3, the executionary caspase whose activation proceeds to apoptosis. Repulsion of apoptosis results in cancer [71]. Apoptosis is mediated by the proapoptotic proteins including the Bax, Bad, Bim, Bak, Bik, Bid and the antiapoptotic proteins including the Bcl-W, Bcl2, Bcl-Xl and Mcl-1. Alterations in the ratio of these proteins result in the activation or inhibition of apoptosis cascade [72].

Metastasis or tumour spread is the major reason behind cancer - related deaths [73]. The process involves the disintegration of the membrane, detachment of tumour cells from the primary tumour, migration to distant sites and formation of secondary tumours [74]. The matrix metalloproteases (MMPs) are critical players in metastasis. Hence, the current research is focused on the inhibition of MMPs thereby preventing the spread of cancer.

Cancer is always accompanied by inflammation. In lung cells, ROS are generated in response to environmental factors. Impaired clearance of ROS causes damage to the lung cells [75]. Earlier studies have shown the constitutive activation of NFkB-p65 in lung cancer [76]. Moreover, the conventional therapies employed in the management of the disease also activate NFkB signaling resulting in drug resistance. The activation of Rel A (p65) form of NFkB is utilized as a biomarker in prognosis cancer [77].

In normal healthy cells, the survival, metabolism and proliferation are regulated by the PI3K/Akt/GSK3 β pathway. Deregulation of this pathway results in uncontrolled cell division thus resulting in cancer [78]. Further, PI3K/Akt cascade can be targeted to identify drugs for NSCLC [79],[80]. Current anticancer therapies target any one component of this pathway to combat cancer [81]. This resulted in developing PI3K inhibitors such as Wortmannin and its derivative LY294002. They were the first generation PI3K inhibitors that entered the market [82], [83]. But the poor drugability and toxicity induced by them in clinical trials caused their withdrawal [84].

The first PI3K inhibitors approved for treatment included the temsirolimus (renal cancer) and everolimus (Breast, renal, endocrine and lung cancers) [85],[86].

Followed by this a series of compounds are undergoing various phases of clinical trials and these include the Buparlisib [87], Pictilisib [88], PX-866 [89], GSK2126458 [90], BEZ 235 [91] and Alpelisib [92].

Based on these reports, it is essential to look for alternate sources of anticancer drugs. Plant-based phytochemicals play a major role in the discovery of new drugs. Phytochemicals activate the immune system, detoxify the dietary compounds from becoming carcinogens, reduce inflammation, relieve cells from oxidative stress, trigger programmed cell death, and prevent the mechanism of carcinogenesis. In terms of red alga *G.acerosa*, few studies have been carried out to determine its therapeutic potential however, the isolation of its bioactives and determination of their anticancer activities are not yet done. Hence, the current study is focused on isolation and characterization of compounds from *G.acerosa* and testing their efficacy on cancer. The study also aimed to explore the pathways and genes critical for controlling lung cancer by using the extract and isolated compounds from *G.acerosa*.

Working Hypothesis

Isolation, characterization and testing of novel anticancer compounds from marine red algae are of paramount importance in search for new therapies for cancer.

Aim

The main aim of the study was to isolate and characterize the phytochemicals from *G.acerosa* and investigate their antioxidant, anticancer, antimetastatic and anti-inflammatory activities under *in silico*, *in vitro* and *in vivo* conditions.

Objectives

- To isolate and characterize the bioactive compound(s) from *G.acerosa*.
- To determine the antioxidant, anticancer, antimetastatic and anti-inflammatory activities of the crude extract and isolated bioactive compounds by *in silico* methods and under *in vitro* and *in vivo* conditions.
- To determine the efficacy of the crude extract and isolated bioactive compounds on apoptosis, cell survival and inflammation.
- To determine the toxicity of the algal extract in Zebrafish (*in vivo*) and utilizing Lab-on-chip technology (*in vitro*).
- To determine the efficacy of the crude extract and isolated bioactive compounds on lung, colon and liver cancer in tumour induced Zebrafish models.
- To identify the mechanism of action of *G.acerosa* extract and isolated compounds in different types of cancer cells.
- To identify the pathways and genes critical in controlling lung cancer by utilizing cell lines and tumour induced Zebrafish.