



Purification, Fractionation of Terpenoid Extracts from Selected Red Algae and Analysis of Its Cytotoxicity Against A549, HeLa and HepG2 Cell Lines

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Abstract

Seaweeds contain a pool of bioactive compounds that had a broad range of biological activities, including anticancer activity. The presence of these active compounds in seaweeds is highly evident of their pharmaceutical potential. Various compounds extracted from seaweeds have been shown to eradicate or slow the progression of cancer. In the present study, the effect of purified terpenoid fractions from three red seaweeds (*Hypnea musciformis*, *Kappaphycus alvarezii* and *Gracilaria dura*) against three human cancer cell lines (A549, HeLa and HepG2) cytotoxicity was evaluated. The crude methanolic extracts of the selected red algae were first subjected to column chromatography followed by GC-MS analysis. The analysis of the purified fraction of *H. musciformis* revealed the presence of 8 major peaks of terpenoids whereas *K. alvarezii* and *G. dura* displayed 12 and 4 major peaks of terpenoids respectively. Further, the cytotoxicity of the purified fractions was carried out using MTT assay. Morphological anomalies were examined using phase contrast microscopy. All the purified terpenoids extracts revealed antiproliferative potential against the three cancer cell lines in a dose and duration dependent manner. *G. dura* terpenoids fraction showed significant inhibition as compared with the other red algae. The microscopic visuals showed outstanding morphological anomalies on the treated cancerous cell lines. Thus, it may be concluded that the terpenoids extract from the three red algae showed remarkable cytotoxicity against the investigated tumor cell lines which further confirms their antiproliferative potentialities.

Keywords

Methanolic, terpenoids, cytotoxic, GC-MS, cancer cell lines, MTT.

INTRODUCTION

Herbal alternate to the synthetic medicines is a promising approach for treating life threatening diseases like cancer. The proven low side effects of bioactive compounds isolated from natural resources established this concept and search for new drug molecules. The search for anticancer agents from plant sources started in the earliest 1950s with the discovery and development of vinca alkaloids such as vinblastine and vincristine and also the cytotoxic podophyllotoxins [1]. Among the various natural resources being exploited to yield anticancer products, marine seaweeds are the latest additions. Seaweeds are extensively used as functional food and medicinal herbs, and have a long history of use among Asian countries [2]. Many seaweeds have long been used for the treatment of cancer either in crude or partially purified forms. These studies have indicated that seaweeds constitute a promising source of novel compounds with potential therapeutic power. In particular, red algae have been considered as a potential source of new bioactive compounds [3]. The seaweeds contain several phytochemicals such as carotenoids, terpenoids, flavonoids, polyphenols, alkaloids, tannins, saponins, minerals, etc. that have been extensively used the drug and pharmaceutical industry [4]. These phytochemicals possess antioxidant activities, which can be used for the treatment of many diseases, including cancer. Triterpenic acids exhibit various biological and pharmacological activities, including anti-inflammatory, antimicrobial, antiviral, cytotoxic, and cardiovascular effects [5].

During the past 20 years, thousands of novel compounds and their metabolites with diverse biological activities ranging from antiviral to anticancer have been isolated from various marine sources [6]. The marine pharmacy currently holds more than 35,000 marine derived biological samples, with approximately 150 compounds as cytotoxic against the tumour cells. Compounds with antioxidant, antiviral, antifungal and antimicrobial activities have been detected in brown, red and green algae [7]. The environment in which seaweeds grow is harsh as they are exposed to a combination of light and high oxygen concentrations. These factors can lead to the formation of free radicals and other strong oxidizing agents. However, seaweeds seldom suffer any serious photodynamic damage which implies that they possess some protective defence mechanisms. Seaweeds have received significant attention for their potential as natural antioxidants. Many studies have documented

regarding the high antioxidant capacity of a range of edible seaweeds. The polysaccharides and peptides compounds were isolated from macro algae have become a substance of great interest for cancer therapy. The mechanisms of their anticancer activity are related to their ability to suppress the growth of cancer cells [8]. Previous studies have proved that seaweeds have been reported to reduce the risk of cancer in animal studies, via their anticell proliferation and antioxidant activity [9, 10].

Many investigations have demonstrated that a high dietary intake of natural phenols such as flavonoids [11] are strongly associated with longer life expectancy, reduced risk of developing some chronic diseases, and various types of cancer [12]. Since, seaweeds are known to contain a wide variety of bioactive compounds they may offer a rich source of new drugs with potentially lower toxicity. To date, research on anticancer activity of terpenoids from red algae species is rather limited [13]. In this juncture, the present study was aimed to test the *in vitro* cytotoxic effects of the purified terpenoids from three selected marine red algae against three cancer cell lines.

MATERIALS AND METHODS

The marine red algae such as *Hypnea musciformis*, *Kappaphycus alvarezii* and *Gracillaria dura* was collected during March 2018, from the Mandapam coast (latitude 9° 17' N, longitude 79° 22' E), Gulf of Mannar. Identity was confirmed by referring algal flora and substantiated by matching with samples of CMFRI, Kochi. Initially, 50 g each of dried algal powdered samples were subjected to Soxhlet extraction with 250 ml of methanol. The extraction was repeated 2 to 3 times. The extract was filtered through What man filter paper No.1 and kept at room temperature for evaporation. Fractionation of the sample was done by silica gel Column Chromatography (CC) using petroleum ether: ethyl acetate solvent combinations. The purified fraction was quantified for the presence of terpenoids and further analysed by GC-MS.

GC-MS analysis

For GC-MS analysis, the sample was injected into a HP-5 column (30 m X 0.25 mm i.d with 0.25 μ m film thickness), Agilent technologies 6890 N JEOL GC Mate II GC-MS model. The chromatogram and the mass spectrum of the unknown components were compared with the spectrum of the known components stored in the NIST library.

MTT assay

The invitro viability of cell lines were determined by MTT assay on the three cell lines Hep G2 (Liver

Hepatocellular Carcinoma), HeLa (Cervical carcinoma), A549 (Lung cancer) cells. MTT is a colorimetric assay that measures the reduction of yellow 3-(4, 5dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. Since reduction of MTT can only occur in metabolically active cells i.e., the level of activity is a measure of the viability of the cells [14]. The percentage of growth inhibition was calculated using the formula:

$$\% \text{ of viability} = \frac{\text{Mean OD Samples}}{\text{Mean OD of control group}} \times 100$$

STATISTICAL ANALYSIS

All the data were mean $\pm$ SD of six independent experiments. Significant level was noted as $p < 0.05$.

RESULTS AND DISCUSSION

The crude methanolic extracts of *H. musciformis*, *K. alvarezii* and *G. dura* were purified by column chromatography. Each fraction was eluted using various petroleum ether and ethyl acetate solvent combinations. Optimal fractions were eluted using 95:5 solvent combination from *H. musciformis*, 50:50 solvent combination of *K. alvarezii* and 90:10 from *G. dura*. The resultants showed significant amount of terpenoids as detected by GC-MS spectra technique. Parallely, the fractions eluted by column chromatography were subjected to thin layer chromatography for confirming the presence of terpenoids. Retention time and the relative abundance of each compound were recognized by GC-MS. The analysis of the 95:5 purified fraction of *H. musciformis* revealed the presence of 8 major peaks of terpenoids compatible with their fragmentation patterns (Fig:1). The 50:50 purified fraction of *K. alvarezii* showed the presence of 12 major peaks (Fig: 2) and 90:10 fraction of *G. dura* shows 4 major peaks of terpenoids (Fig: 3). Table I summarizes the terpene components, retention time (RT), molecular weight (M.W.), molecular formula (M.f) and concentration percentage of *H. musciformis*, *K. alvarezii*. and *G. dura*.

There was a significant difference in terpenes content between the studied species. Some of the terpene compounds in the selected seaweeds showed marginal differences in their retention time. This may be attributed by the sequence of increasing polarity of the separated compounds that was detected via GC-mass detector of the species. Hexadecanoic acid, methyl ester was common among the three species and the fraction showed difference in their retention time. This terpenoids

fraction was proved for its many biological properties.

Terpenoids have multiple functions like inhibition of tumour proliferation via apoptosis triggered activity, and cation channel regulation. Three bioactive components such as alpha-amyrin, beta-amyrin and lupeol from *Dandelion* root extract have been reported as anticancerous compounds [15]. Eman et al [16] also reported the presence of terpenoids 4, 8, 12, 16 tetramethyl heptadecan-4-olide; 2, 6, 10, 14 Tetra methyl hexadecane from the filamentous green algae *Spirogyra*. Marine algae of the genus *Laurencia* was first noted for the terpenoids content. Similarly, the brown algae *Dictyota*, showed a wealth of terpenes [17]. Alpha and beta-amyrins were two promising bioactive natural products (pentacyclic triterpenes) that have been shown to exhibit various pharmacological activities such as anti-inflammatory, antihyperglycemic, antioxidant, gastro protective, hepatoprotective and hypolipidemic effects at nontoxic concentrations. Ghazala and Shameel [18] identified diterpene phytol from brown algae. Gupta and Abu Ghannam [19] reported that diterpenoids and sesquiterpenoids have cytotoxic, antiviral and algicidal activities. *G. dura* showed profound % of terpenoids such as Hexadecanoic acid methyl esters (15.58), n-Hexadecanoic acid (80.78), 11-octadecanoic acid, methyl ester (80.78) and Phytol (3.65).

The *in vitro* cytotoxicity of purified terpenoid was performed against three human cancer cell lines namely lung (A-549), liver (Hep-G2) and cervical (HeLa) cell lines. Cytotoxicity of marine algal extracts was evaluated by growth inhibition assay. The growth inhibited cells were stained to analyse the apoptotic cell death in time and dose depended manner in the cultures. The obtained results suggest that purified terpenoids fractions from all the three algae caused irreversible cell damages in the cultured cells. *H. musciformis* vs lung (A-549) cell line showed 39.5% growth inhibition. In the case of liver (Hep-G2) the extract showed 34.7% inhibitory activity where as in case of HeLa cell line terpenoid extract showed maximum activity i.e., 50%. Similarly, the *K. alvarezii* vs lung (A-549) cell line showed 47.1 % growth of inhibition, 34.2 % growth of inhibition against Hep G2 and 36.7 % growth of inhibition against HeLa cell lines. *K. alvarezii* showed greater activity against the lung cancer cell line (A549) in comparison with other two cell lines. The terpenoids extract of *G. dura* showed maximum inhibitory activity in comparison with other two red algae i.e. 48.28 % of growth inhibition against lung cancer cell line, 43.07 % and

52.59 % of growth inhibition against Hep G2 and HeLa cell lines respectively. HeLa cell lines were most sensitive, whereas HepG2 was the most resistant against the terpenoids in the assay. These results confirmed that the seaweed extracts were selectively inhibited the growth of a particular cell type or tumour type.

In the report of Harada et al. [20], 47 species of alga exhibited strong cytotoxic activity against L1210 cells. They also showed low cytotoxicity against normal cells. Such selective activity was also conspicuous in other seaweeds reported in their experiment. All these results together with this study suggests that the active substances interact with special cancer-associated receptors or cancer cell metabolic pathway, thus triggering to cell death.

Morphological alteration of malignant cell lines upon exposure using terpenoid extract was observed under phase contrast microscope. The cells indicated prominent abnormalities after exposure to the terpenoid extract. The microscopic visuals show that the terpenoid extract of *G. dura* have outstanding effect on treated cancerous cell lines in comparison with other two red algae. Lesser activity was showed by the terpenoid extract of *K. alverzi*. The number of dead cells increased correspondingly with increasing concentration of the extract. At high extract concentration, enlargement and clumping of the cells were conspicuously observed. 39%-50% of the cells showed membrane blebbing (demonstrated with small protrusions of the membrane) and ballooning were apparent in the cells.

Induction of apoptosis is a useful approach in cancer therapies. In apoptotic cells, several cellular and biological anomalies like cell shrinkage, DNA fragmentations and activation of the caspase cascade were documented [21]. The presence of apoptotic cell death could also be noticed in the extract treated cells (Fig 4, 5, 6). Cells also showed extensive vacuolation in the cell cytoplasm, indicating autophagy like mechanism of cell death. At highest concentration (100 µg/mL) the cells became rounder, shrunken and showed signs of detachment from the surface of the wells denoting cell death. In fact, no cytotoxicity was noticed with human blood lymphocytes (as non-malignant control cells).

Currently, much attention has been paid for the screening of biologically active compounds from marine organisms. Among those, seaweeds are considered to be attractive sources because of their biodiversity, safety and as they have long been used in traditional Asian foods. MTT reduction is usually carried to study mitochondrial dehydrogenase activity as a cytotoxic test for a variety of secondary metabolites. Therefore, the mode of action of terpenoid extract from the three red algae may be via changing the enzymatic activity of mitochondria and initiate a preliminary injury that leads to cell death.

Cancer increased the tissue oxidative stress and it was observed that *E. cottonii* extract administration to rat significantly improved the oxidative status [22]. This improved oxidative status contributed to the *in vivo* tumour suppression response. The cell alleviated oxidative stress was either by repairing the damaged nucleotides and lipid peroxidation and products or by directly reducing the pro oxidative state.

Furthermore, it was also documented that polyphenolic derivatives can cause damage in the mitochondrial membrane since they provoke depolarization of the mitochondrial membranes by decreasing the membrane potential [23] and also alter the fluidity of membranes which become abnormally permeable. Although, several reports have suggested that crude seaweed extracts have antiproliferative activity in cancer cell lines, via their antioxidant activity. Water-soluble polysaccharides, such as laminarans and fucoidans, are proven anticancer substances extracted from seaweeds [24]. It was reported that the red algae methanol extract of *Gelidium acerosa* and *Acanthophora spicifera* possesses anticancer activity against Dalton's Ascitic Lymphoma (DAL) cells bearing mice [25]. Thus, the differential inhibition showed by the terpenoid extracts of all the three red algae was justifiable. Parker et al. [26] have also reported that methanolic extracts of various kelps exhibited dose-dependent inhibition of the growth of human gastric (AGS) and HT-29 colon cancer cells.

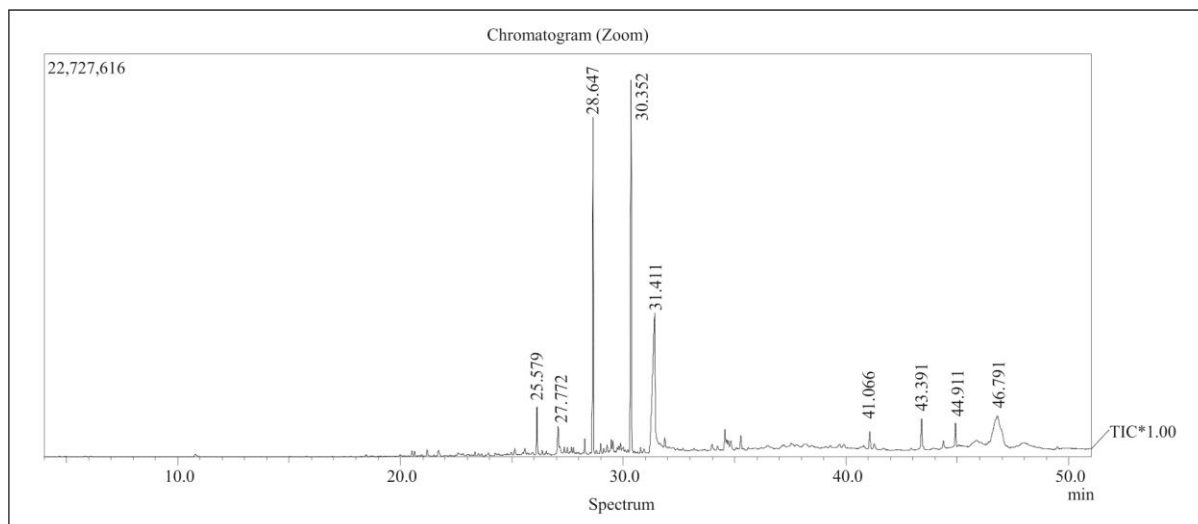


Fig 1: GC- MS spectra showing terpenes composition of *Hypnea musciformis*

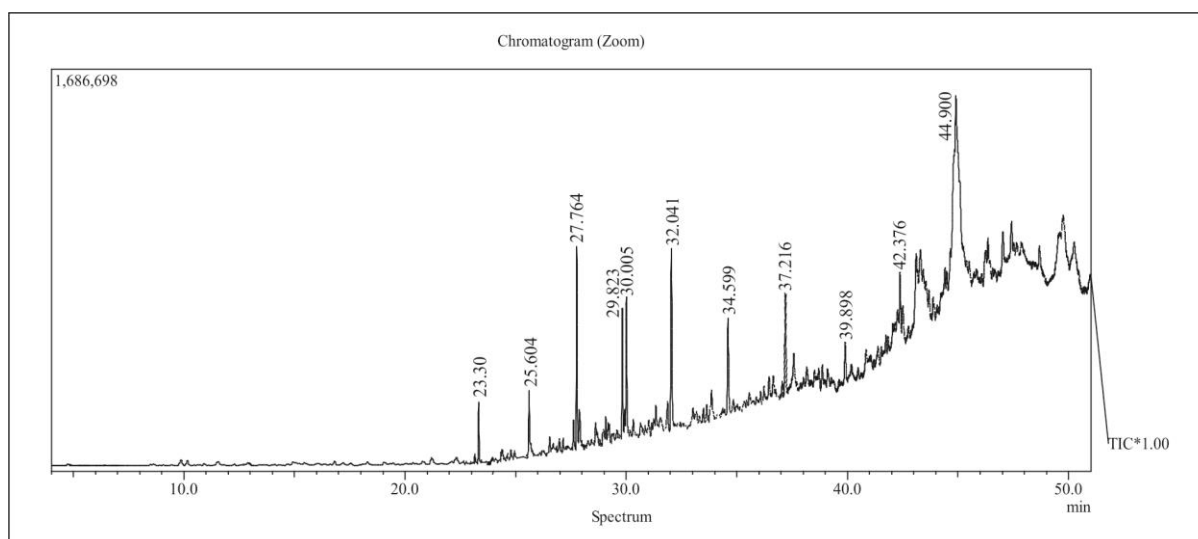


Fig 2: GC-MS spectra showing terpenes composition of *Kappaphycus alvarezii*

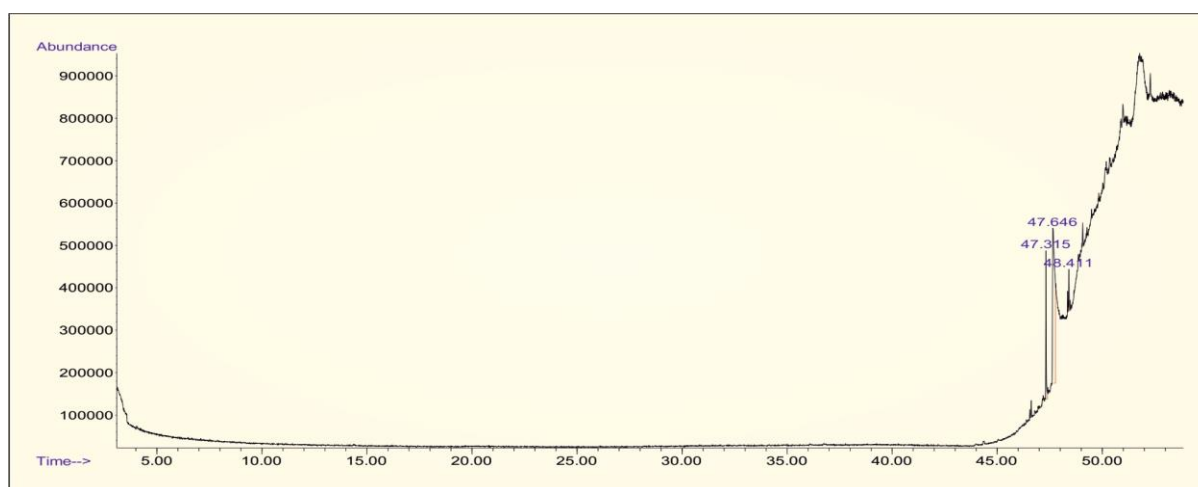


Fig 3: GC-MS spectra showing the terpene composition of *G. dura*

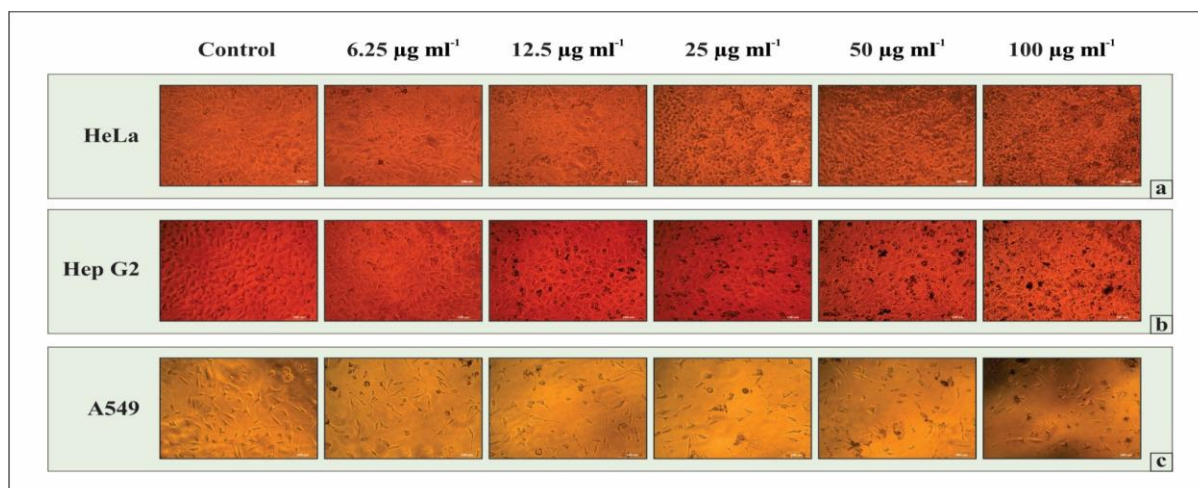


Fig 4: a,b,c showing the anticancerous effect of terpenoid extracts of *H. musciformis* against various cell lines

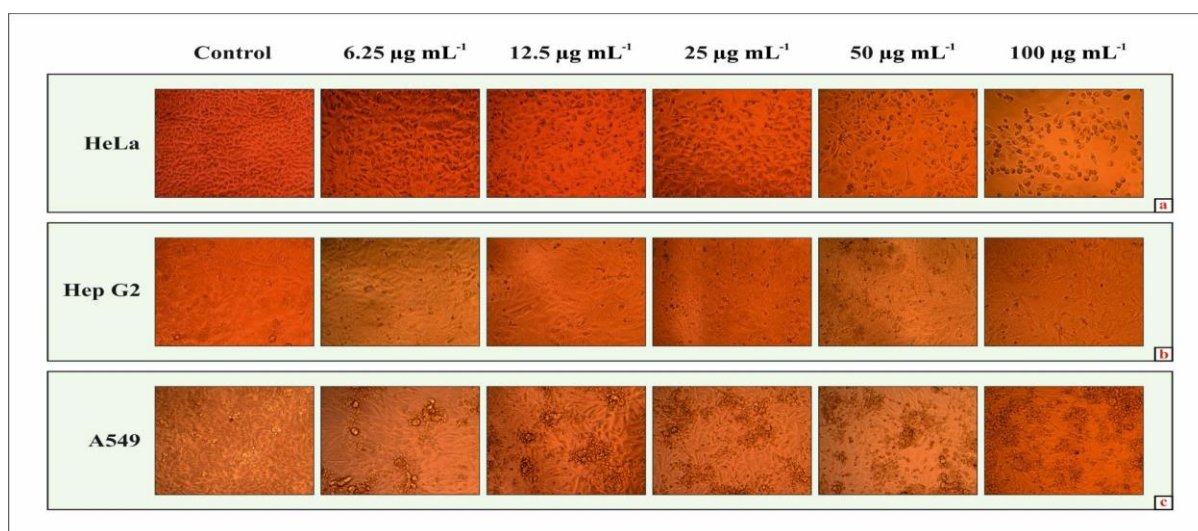


Fig 5: a,b,c showing the anticancerous effect of terpenoid extracts of *K. alvarezii* against various cell lines

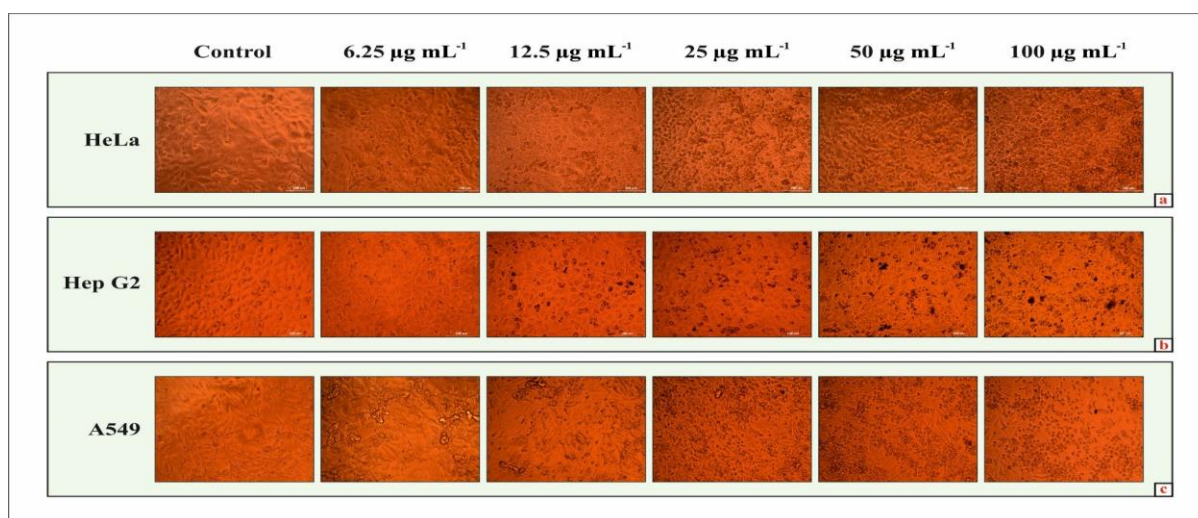


Fig 6: a, b, c showing the anticancerous effect of terpenoid extracts of *G. dura* against various cell lines

Table.1: Terpene components identified in *H. musciformis*, *K. alvarezii* and *G. dura* by GC-MS.

Compound Name	Molecular weight	Molecular formula	<i>H. musciformis</i> Area. %	<i>K. alvarezii</i> Area. %	<i>G. dura</i> Area %
Hexadecane	C ₁₆ H ₃₄	226	-	1.64	-
Eicosane	C ₂₀ H ₄₂	282	0.54	5.91	-
Hepta Decane	C ₁₆ H ₃₄	226	-	2.43	-
Octadecane	C ₁₈ H ₃₈	254	—	5.30	-
Heneicosane	C ₂₁ H ₄₄	296	0.27	3.07, 4.48, 1.85, 1.35, 2.32	-
Tricosane	C ₂₃ H ₄₈	324	—	—	-
2- Pentadecanone	C ₁₄ H ₂₈ O ₂	228	18.49	—	-
Hexadecanoic Acid, Methyl Ester	C ₁₇ H ₃₄ O ₂	270	21.82	3.27, 3.72	15.58
N-Hexadecanoic Acid	C ₁₆ H ₃₂ O ₂	256	21.30	—	80.78
Hexadecanoic Acid,Ethyl Ester	C ₁₈ H ₃₆ O ₂	284	0.50	—	-
Beta Amyrin	C ₃₀ H ₅₀ O	426	—	47.17	-
Heptadecanoic Acid,Methyl Ester	C ₁₈ H ₃₆ O ₂	284	0.13	—	-
11- Octadecanoic Acid,Metyl Ester	C ₁₈ H ₃₆ O ₂	284	0.33	—	80.78
Phytol	C ₂₀ H ₄₀ O	296	-	-	3.65

Table 2. Cytotoxicity analysis of *H. musciformis*, *K. alvarezii*, *G.dura* against selected cancer cell lines after 48 h

Sample Concentration (µg/mL)	Percentage Viability of <i>H. musciformis</i>	Percentage Viability of <i>K. alvarezii</i>	Percentage Viability of <i>G. dura</i>
HeLa			
Control	100.00	100.00	100.0
6.25	76.78	85.88	97.99
12.5	72.39	74.38	86.05
25	64.95	71.81	72.58
50	60.60	66.60	53.07
100	50.24	63.28	47.41
Hep G2			
Control	100.00	100.00	100.00
6.25	86.62	94.09	87.37
12.5	77.02	90.20	81.91
25	73.07	80.56	79.86
50	67.88	77.98	74.95
100	65.30	65.80	56.93
A549			
Control	100.00	100.00	100.00
6.25	92.78	98.33	97.93
12.5	85.06	85.04	85.44
25	81.02	76.12	74.06
50	76.89	68.38	64.19
100	60.54	52.81	51.72

CONCLUSION

The present study, showed that the purified terpenoid extracts from *H. musciformis*, *K.alvarezii* and *G. dura* could inhibit the growth of cancer cells in varied % and could induce apoptosis in cervical cancer, lung cancer and liver cancer cell lines in time and dose depended manner. Thus, the analysed

marine red alga seaweeds possess abundant source of potential complementary and alternative secondary metabolites like terpenoids for prevention and treatment of cancer.

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