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Antioxidant and bioactive properties of the various solvent extracts of Kappaphycus alvarezii (Doty) cultivated in the Gulf of Mannar coast

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ABSTRACT

The aim of the present study is to evaluate various solvent's efficiency in extraction (aqueous, 40% ethanol, 60% ethanol, and 80% ethanol) for FTIR, total phenolic content, total flavonoid content, DPPH antioxidant activity, and bioactive properties. The FTIR analysis in the seaweed extracts confirms the presence of polyphenols and flavonoid content and it was predominant in the ethanolic extract. The total phenolic content ranges from 9.87 ± 0.073 -12.82 ± 0.067 mg of GAE.g -1. Generally, ethanolic extract (E60) showed enhanced phenolic content, flavonoid content (4.44 ± 0.07 mg of QE.g -1), DPPH assay (IC50 6.50 ± 0.07mg.ml-1). The best solvent extract (E60) was selected and possesses significant antidiabetic properties as identified by α-Amylase (IC50 0.089 ± 0.01 mg/ml), α-glucosidase (IC50 0.085 ± 0.01 mg/ml), and anti-inflammatory activity (IC50 0.10 ± 0.01 mg /ml). The MTT assay using E60 seaweed extract (0.5 - 2.5mg/ml) conducted against MCF-10A and MCF-7 cells showed maximum inhibition activity of 65.75% and 34.3% respectively. The present findings highlighted that E60 solvent is used for obtaining antioxidant-rich extracts and bioactive properties.

KEYWORDS

Seaweed Extract, Antioxidant, Antidiabetic, Anti-Inflammatory, Cytotoxicity

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Nowadays, there is an increased demand for consumption of the compounds rich in high bioactive properties which have the ability to reduce lifestyle-associated diseases such as cardiovascular diseases, cancer, etc (Pérez-Jiménez *et al*., 2008). Several commercially available synthetic antioxidants like Butylated hydroxy anisole (BHA).

Butylated hydroxytoluene (BHT) and tert butylhydroquinone have some restrictions to being used in foods due to their carcinogenic nature found in some toxicological studies in rats. This has stimulated consumer's interest in natural food additives resulting in the development of alternative antioxidants derived from natural origin (Huang and Wang, 2004).

In this context, seaweed is considered a natural source of antioxidants as it doesn't create any oxidative damage when exposed continuously to free radicals and acts as a strong oxidizing agent due to the structural component of seaweeds (Plaza *et al*., 2008). Besides, Seaweeds are a rich source of nutrients and bioactive compounds such as proteins, lipids, minerals, polyphenols, sulfated polysaccharides, and organic acids and the presence of these compounds provide wide applications in foods and pharmaceutics based on their antioxidant, antibacterial, antiviral, antitumor and antifungal properties (Cornish and Garbary, 2010; Gupta and Abu-Ghannam, 2011). The development of cancer is due to the progressive attack of reactive oxygen species on DNA in cells to reduce the cancer risk seaweed consumption helps to increase endogenous antioxidant enzymes in vivo such as superoxide dismutase, glutathione peroxidase, and sometimes also catalase activity (Corsetto *et al*., 2020; Korivi *et al*., 2019; Bayro *et al*., 2021). Based on its properties it has a wide range of potential applications in the areas of functional food s, Pharmaceuticals, biotechnology, etc. (Rajauria *et al*., 2013).

Kappaphycus alvarezii (Doty), an economically important seaweed that belongs to the phylum Rhodophyta commonly known as Eucheuma cottonii and the culture of the seaweed already began in Mandapam, Tamilnadu during the year 1995- 1997. The seaweed is primarily utilized in the production of kappa carrageenan which is used in several products such as gels, meat, and dairy products, pet foods, toothpaste, air freshener gels, and immobilized biocatalysts (da Costa *et al*., 2017, Hong *et al*., 2010, Mendoza *et al*., 2006). Also, *K. alvarezii* is potentially used for dietary fiber, cholesterol reducer, a source of antioxidant, anti-viral, and anti-cancer compounds, and hemagglutination activity (Casais *et al*., 2012). There are several techniques involved in the extraction of seaweed bioactive compounds like conventional extraction, maceration, Soxhlet extraction, microwave extraction, and enzyme-assisted extraction. The efficiency of extracting bioactive compounds mainly depends upon the type of solvent, its polarity and extraction conditions, etc. Among the solvents, ethanol and methanol are considered effective solvents in breaking the cell for extraction of bioactive compounds (Lapornik *et al*., 2005).

In addition, aqueous solutions of ethanol, methanol, or acetone were considered a better solvent than a single solvent system due to their ability in dissolving a wide range of phenols. Ethanol mixtures have acceptability in human consumption models (Tomsone *et al*., 2012). Many researchers have reported the antioxidant properties of both brown and red seaweeds from across the globe (Heo *et al*., 2005). But there are a few shreds of shreds of evidence regarding the comparative assessment of different solvent concentrations in extracting bioactive compounds. The present study was carried out to examine the complete nutritive profile and conventional solvent extraction methodologies in retaining the bioactivity property of the seaweed extract with respect to FTIR analysis, total phenolic content, total Flavanoid content, DPPH activity, antidiabetic, ant-inflammatory, and cytotoxic activity. The study helps us to produce high bioactivity rich seaweed which will be incorporated in functional foods that provide with healthy diet with enriched nutrition.

Materials and Methods

Raw Materials Collection

Fresh seaweed *Kappaphycus alvarezii*, from mandapam, was handpicked from the cultured sites brought into the laboratory in iced conditions, and immediately washed to remove the epiphytes, sand, and dust particles. After washing the seaweeds were spread on a blotting paper to remove the water from the seaweed. The seaweeds were dried in a cabinet drier (Thermoline, NSW, Australia) at 40 ± 2ºC for 12 hours. The dried seaweed was made into powder by passing through a 250 µm mesh sieve.

Proximate Composition

The proximate composition of seaweed powder was determined in triplicates .Moisture content was analyzed using the hot air oven method (AOAC, 950.46, 2016), Total fat by Soxhlet method (AOAC, 991.36, 2016), total crude protein by Kjeldahl distillation method with the conversion factor of 6.25 (AOAC, 928.08, 2016), total ash by heating in a muffle furnace (AOAC, 938.08, 2016), carbohydrate was calculated by phenol sulfuric acid method (Dubois *et al*., 1956) and fiber analyses (AOAC, 2000) were also performed in the study.

Antioxidative Properties of Seaweed Extract
Preparation of Seaweed Extract

The extraction of seaweed was done by using four different solvents Water, 40% ethanol (E40), 60% ethanol (E60), and 80% ethanol (E80). 10g of seaweed powder was mixed with 100 ml (1:10 w/v) of the respective solvents. The mixture was kept in a magnetic shaker at 650 rpm for 24 hours. The supernatant was taken after centrifugation at 2096 rpm for 15 minutes. The residues were carried out for the second extraction under the same conditions. The supernatant thus obtained was pooled together and condensed in a vacuum at 40 ºC using a rotary evaporator. The extracts were lyophilized and the dried extract and stored at 4 ºC until further study.

Fourier Transform Infrared Spectroscopy (FTIR) Analysis

FTIR Spectroscopy was carried out by using FTIR Spectrophotometer (ATR-FTIR, Model P-4600, Thermo Scientific, USA). The dried seaweed extracts (Aqueous, E40, E60, E80) were mixed with KBR powder (Potassium Bromide) in a ratio of 1:9 and ground well. The powder was added to the collar after assembling the die and given a hydraulic press of about 100 kg/m^3 for the formation of the pellet. The pellet was taken out from the collar after disassembling the die. The spectrum was then recorded after placing the pellet on the sample holder and the signal was obtained in 32 scans at a resolution of 4 cm^{-1} from a range of 600-4000 cm^{-1}.

Estimation of Total Phenolic Content

The total phenolic content determined in seaweed extract was carried out according to the method of (Slinkard and Singleton, 1977). The solution (0.5 ml) from the extract was mixed with 0.5 ml of distilled water followed by 0.5 ml of Folin Ciocalteu reagent (1:1 with water) and 2.5 ml of 2% sodium carbonate solution was added. The reaction mixture was mixed thoroughly and placed in the dark for 40 min and the absorbance was recorded at 725 nm using a double-beam spectrophotometer (Model UV-1800, Shimadzu, Kyoto, Japan). The total phenolic content was calculated from the standard curve of gallic acid (0-0.1mg/ml) and expressed as mg gallic acid per gram of dry extract after blank subtraction.

Blank for each extract was prepared in the same manner, except that distilled water was used instead of Folin–Ciocalteu reagent.

Estimation of Total Flavonoid Content

The determination of flavonoid content in seaweed extracts was analyzed according to (Kumar *et al*., 2020). The seaweed extracts (0.5 ml) were mixed with 4 ml of distilled water and 0.3 ml of 5% $NaNO_2$. Followed by the addition of 0.3 ml of 10% $AlCl_3$, and 2 ml of 1 M NaOH and made the volume up to 10 ml using distilled water. The absorbance was measured at 506 nm in which qurectin was used as the standard and the results were expressed as mg of querctin equivalent/g of extract

Estimation of DPPH Radical Scavenging Activity

The antioxidant properties were determined by DPPH scavenging potential in which ascorbic acid was used as a positive control according to Blois 1958. The 2 ml of seaweed extract (2-8 mg/ ml) added with 2 ml of 0.16 mM methanolic DPPH and the mixture was incubated at room temperature for 30 minutes. The development of yellow color in the reaction mixture indicates the positive scavenging activity. The absorbance was determined at 517 nm and calculated by the equation:

$$\text{DPPH Radical Scavenging Activity (\%)} = \left(\frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \right) \times 100$$

Bioactive Properties
In vitro Antidiabetic Activity
α-Amylase Inhibition Assay

The extract with high antioxidant properties was added with 100 µl of 0.02 M sodium phosphate buffer (pH 6.9) and 100 µl of α-amylase solution (4.5 Units/ ml/ min) which were preincubated at 25°C for 10 min (Worthington, 1993). Then, 100 µl of 1% starch solution was added and incubated at 25°C for 30 minutes and the reaction was stopped by the addition of 1.0 ml of dinitro salicylic acid reagent. The reaction mixture was then incubated in a boiling water bath for 5 minutes and then cooled to room temperature, it was diluted (10-fold) with distilled water and the absorbance was measured at 540 nm. The control which contains buffer instead of extract and the percentage of α-amylase enzyme inhibition were calculated.

α-Glucosidase Inhibition Assay

The different concentration of the E60 extract was taken with 100 µl of 0.1 M phosphate buffer (pH 6.9) and 100 µl of α-glucosidase solution (1 Unit/ml/min) and preincubated at 25°C for 5 min (Worthington, 1993). Then, 100 µl of p-nitrophenyl-α-D-glucopyranoside (5 mM) was added and incubated at 25°C for10 min. After the incubation period, the absorbance readings were recorded at 405 nm and allegorized to control that had 100 µl of buffer in place of the sample extract. The results were expressed on a percentage basis.

In vitro Anti-inflammatory Activity

The anti-inflammatory activity of the given sample was studied by using the inhibition of albumin denaturation technique which was studied according to (Mizushima *et al*., 1968 and Sakat *et al*., 2010) followed by slide modifications. In the different concentrations of ethanolic seaweed extracts (10-150 µg/ ml) with 1% aqueous solution of bovine albumin fraction, the pH of the reaction mixture was adjusted using a small amount of 1N HCl. The sample extracts were incubated at 37 ºC for 20 minutes and then heated to 51 º C for 20 minutes, after cooling the samples the turbidity was measured at 660nm by UV-Visible Spectrophotometer. The Percentage inhibition of protein denaturation was calculated

In vitro Cytotoxicity Assay

The MTT assay was analyzed according to Mossman, 1983. The human breast epithelial cells (MCF 10-A) and cancer cells (MCF-7) were maintained in DMEM medium, supplemented with 10% Fetal Bovine Serum, at 37ºC in a humidified atmosphere with 5% CO_2. The MCF 10-A and MCF-7 cells were plated in 96 well flat bottom tissue culture plates at a density of approximately 1.2 X 10^4 cells/ well and allowed to incubate overnight at 37ºC. The medium was discarded and cells were incubated with different concentrations of the seaweed extracts (125, 250, 375, 500 and 625 µg/ ml) for 24 hours. After the incubation, the medium was discarded and 100µl fresh medium was added with 10µl of MTT (5mg/ ml). The MTT solution was discarded after 4 hours and 100µl of DMSO was added to dissolve the formazan crystals.

Then, the absorbance was read at 570nm in a microtiter plate reader against cyclophosphamide which was used as a positive control. Medium along with cells (untreated) serves as a control. The MTT cytotoxicity was expressed as the concentration of drug inhibiting cell growth by 50 % (IC50) was calculated after comparing with the control (treated with 0.1 % DMSO). Cell survival was calculated by the following formula:

Viability % = (Test OD/ Control OD) X 100 Cytotoxicity % = 100 – Viability %

Statistical Analysis

All the analyses were run in triplicate and the results were expressed as mean ± standard deviation. The recorded data were processed and analyzed using the statistical software SPSS version 16 which is used to test the significant difference in various solvent extracts by performing a single factor one-way analysis of variance (ANOVA).

Results and Discussion
Proximate Composition

The moisture content determined from fresh *Kappaphycus alvarezii* (Doty) was 86.18 ± 0.32% The proximate composition of seaweed powder in terms of its protein, fat, carbohydrate, fiber, ash content, and energy were found to be 6.20 ± 0.092 %, 1.426 ± 0.061 %, 57.71 ± 0.715 %, 6.70% ± 0.120, 16.3 ± 0.2715 % and 3092 ± 1.525 % (on dry basis) respectively. The presence of cell wall sulfated polysaccharides in seaweed has the ability to accumulate inorganic substances with a high mineral content (Bocanegra *et al*., 2009). Similar results were reported by (Xiren *et al*., 2017) from the seaweed collected from Langkawi and Sabah with moisture content ranging from (86.8 -84.8%), protein (6.2-6.8%), fat (0.9-1.0% fibere (7.8-8.9%), ash (16.3-17.1%). Few reports showed some variations in their proximate compositions with a lower moisture content of 57.35%, the protein content of 3.13% (Mustafa *et al*., 2018) and 78.78 % of lower moisture and higher ash content of 23.25% (Ahmad *et al*., 2012) The variation in seaweed composition is according to the season, age, population, species, geographic location and temperature (Norziah and Ching, 2000).

The fiber-rich foods can have several positive effects in preventing constipation, cardiovascular disease, colon cancer, etc.

FTIR Analysis

FTIR analysis is an important technique in the characterization of various seaweed extract functional groups corresponding to different adsorption bands which are responsible for the adsorption (Prabu *et al*., 2012). In this present study, different solvent extracts (Aqueous, E40, E60, E80%), and the phenolic content functional groups were elucidated in the spectrum shown in Figure 1(a, b, c, d). The peaks obtained in the range from 3200-3800 cm^{-1} correspond to the O-H stretch of phenol groups and 800 - 600 cm^{-1} is for (C-H out of plan bend)aromatic phenols were present in all the seaweed extracts (Kumar *et al*., 2018; Rao and Paria, 2013). The phenolic compounds obtained at 1650-1450 cm^{-1} and 1420-1330 cm^{-1} due to aromatic stretching vibration and O-H in-plane deformation, whereas in the region (1300-1200 cm^{-1}) due to C-O stretching related to phenols but the intensity was high in case of ethanolic extracts (Vazquez *et al*., 2008; Ma *et al*., 2016). The distinctive peaks at 1225.43 cm^{-1} (E40),1262.09 cm^{-1} (E60) ,1249.94 cm^{-1} (E 80) in different proportions of ethanolic extracts is a characteristic feature of flavonoid-based compounds and the disappearance of the peak was observed in case of aqueous extract. Similar results were reported according to (Falcao and Araujo, 2013). Hence, it was concluded that the seaweed extracts were enriched with phenolic compounds.

Antioxidative Properties of Seaweed

Seaweeds are found to be high in phenolic compounds, which have excellent biological properties such as antioxidant and antimicrobial activity (Kuda *et al*., 2005). Several factors have been reported to influence antioxidant compounds extraction, including the nature of the sample, the type of extraction solvent, its polarity, the time of extraction, and the sample size (Prior, Wu, and Schaich, 2005). Only food-grade solvents such as water, ethanol, and aqueous mixtures of ethanol were used in this study to prepare seaweed extracts.

Effect of Solvents in the Extraction Yield

The extraction yield was solely depending upon the solvent polarity, pH, extraction time, and the composition of the sample (Do *et al*., 2014). The most important stages in the isolation of bioactive compounds from plant material is mainly the extraction methods especially optimizing the most suitable solvent for the extraction from the plant material (Azwanida *et al*., 2015). The order of the yield of various solvent extracts of *K. alvarezii* in descending order was: Aqueous (9.26 ± 0.039 %) >40% ethanol (8.67 ± 0.002 %) >60% ethanol (8.13 ± 0.001 %) > 80% ethanol (6.56 ± 0.019 %) shown in table 1. The extraction is efficient due to the presence of water in the organic solvent as it increases the dielectric constant by isolating the bioactive components which are soluble in both water and organic solvents. The maximum biomass can be extracted by the usage of 50% aqueous methanol and ethanol (Monteriro *et al*., 2020). The yield obtained in this study is slightly lower compared to the 80% ethanolic extract of *G. changii* (Chan *et al*., 2014). The yield obtained was higher than the previously reported in various methanolic seaweed extracts, including for *G. edulis* (2.85%), *Eucheuma* sp (3.98 %), and *Acanthophora spicifera* (5.01 %) and (Ganesan *et al*., 2008) and around (10.72 % to 12.99 %) *Kappaphycus alvarezii* (Neoh *et al*., 2016). The variation observed in extraction yield amongst different solvents and different seaweed species may be due to the polarities of different compounds present in the seaweeds and also due to the difference in species (Cho *et al*., 2011; Larsen *et al*., 2013).

Total Phenolic Content

Polyphenolics are the secondary metabolites obtained mostly in plants as it widely deposited in the cell wall whereas the compounds like flavonoids and lignin get deposited in the vacuoles and the compounds associated with several biological activities such as protection from oxidative stress damage (Maadane *et al*., 2015; Neoh *et al*., 2016). The antioxidant capacity of phenolics is mainly due to their redox properties the increase in phenolic content is corresponding to the polarity of the solvent which helps in increasing phenolic solubility (Balange and Benjakul, 2009).

Similarly, ethanolic solvent helps in extracting total phenolic content compared to methanol in Kiam wood extraction based on the polarity (Naczk and Shahidi, 2006). In addition, ethanolic mixtures have acceptability for human consumption models (Tomsone *et al*., 2012). TPC content showed a significant difference (P< 0.05) between aqueous (9.87 mg GAE g^{-1}), 40% ethanolic extracts (10.98 mg GAE g-1) and 80% ethanolic extract (12.70 mg GAE g^{-1}). However, there was no significant difference between 60% ethanol (12.82 mg GAE g^{-1}) and 80 % ethanol (12.70 mg GAE g^{-1}). The values were shown in table 1. Previous reports obtained were obtained in a similar range from 8.66 to 12.97 mg PGE g-1 among different drying methods in methanolic Kappaphycus extracts. In the case of Ulva seaweeds, the content of phenolic compounds varied from 5.08 ± 0.65 to 1.258 ± 0.126 mg GAE g^{-1} (Farsad *et al*., 2014). The values were in accordance with the studies having similar phenolic content reported by (Neoh *et al*., 2016). Diyana *et al*. (2015) exhibited TPC values of *K. alvarezii* in the range from 6.74–17.32 mg GAE/ 100g wet weight sample with maximum intensity using 50% ethanol solvent and (Chan *et al*., 2014) reported around the significant difference between 6.06±0.52 mg PGE g−1 in the aqueous extract to that of 10.83±0.65 mg PGE g−1 in ethanol extract. Thus, the study proved that the increase in total phenolic content is with an increase in solvent polarity and provides good antioxidant properties.

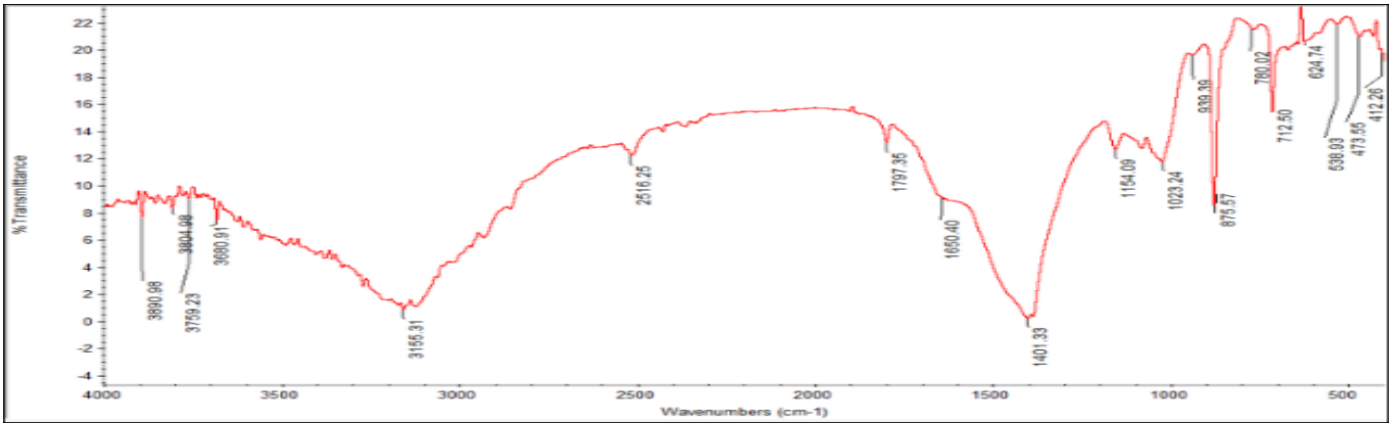
Total Flavanoid Content

Flavonoids are one of the polyphenolic compounds having high redox potential ability and act as reducing agents, hydrogen donors, and singlet oxygen quenchers with metal chelating potential (Velioglu *et al*., 1998). The occurrence of oxidation reactions in the human body causes damage and to prevent these crucial actions antioxidant complex system needs to be incorporated into the body. The antioxidant capacity of flavonoids is obtained due to the conjugation between the flavonoid rings from the hydroxyl group (Zeka *et al*., 2017). The TFC content showed a significant difference (P <0.05) among aqueous extract (2.64mg QE g-1), 40% Ethanolic extract (3.14mg QE g-1), 60% Ethanolic extract (4.412 mg QE g-1) and 80% ethanolic extract (4.19 mg QE g^{-1}) as illustrated in table 1.

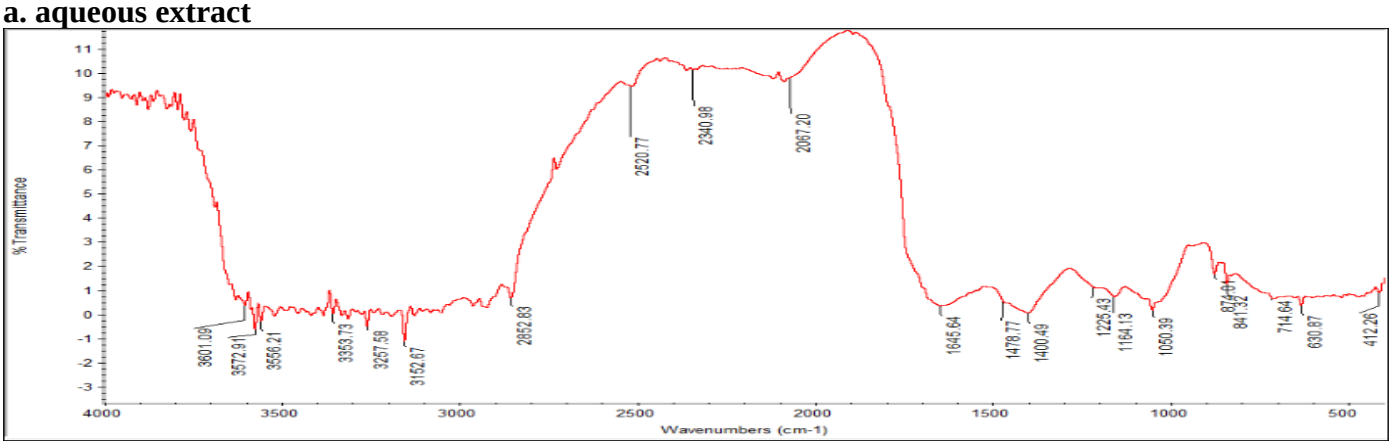
The lowest TFC was produced by the extraction using water, whereas the highest TFC was produced by the extraction using aqueous ethanol. However, among different extracts 60% ethanolic extract showed higher TFC content. Similar trends were obtained with the values corresponding to TPC. Similar findings were reported by (Neoh *et al*., 2016) around 3.10-5.37 mg of QE g^{-1} and (Ling *et al*., 2015) of around 9.83–25.67 mg CE $100g^{-1}$ DW. The extraction using ethanol showed the highest total flavonoid content in *Gracilaria* sp (Sasadara and Wirawan, 2021). The variation in flavonoids may be due to the change in physiochemical characteristics such as salinity etc. The results revealed there is a positive correlation between the total phenolic content and the flavonoid content.

DPPH Radical Scavenging Activity

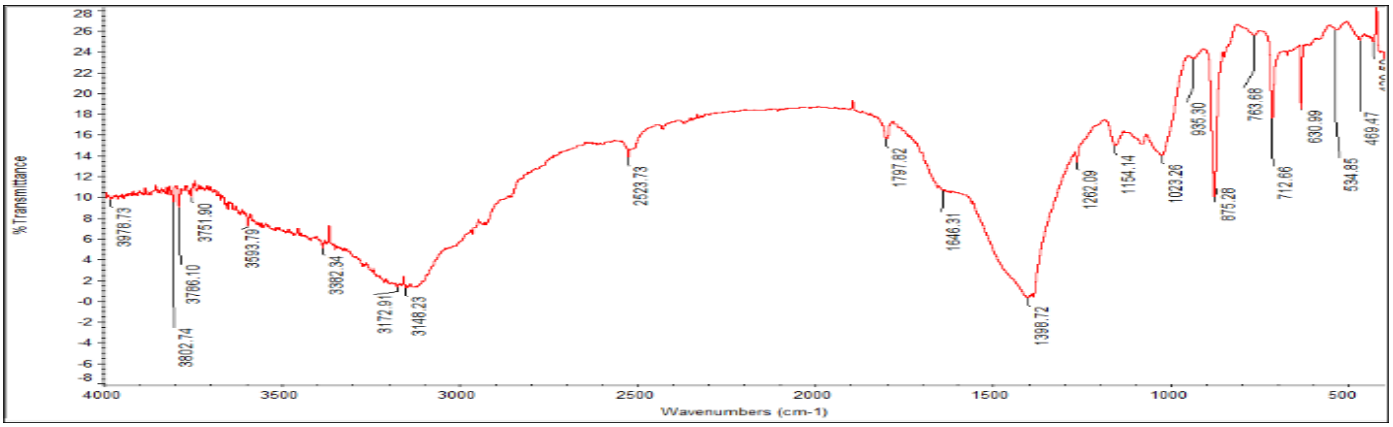
DPPH is a stable free radical, which is scavenged by antioxidants resulting in the donation of an electron or hydrogen atom to the DPPH radical becoming a stable molecule that changes color from purple to yellow (Prior *et al*., 2005). The decrease in absorbance is the consequence of high antioxidant capacity. The variation that occurred among various seaweed extracts regarding radical scavenging activity was probably due to changes in the chemical composition of each extract which corresponds to a significant change in antioxidant activity (Samarth *et al*., 2008). The DPPH assays were represented as IC_{50} and the values were determined by means of plotting DPPH radical scavenging assay extracts against the concentration of extracts shown in table 1. The EC_{50} of various solvent extracts showed significant differences (P<0.05) and the values ranged from 9.62–6.50 mg ml^{-1} with best radical scavenging activity obtained in E60 extracts. Substantially, there has been a sturdy correlation between the scavenging interest and phenolics, suggesting that the polyphenolic compounds present in *K. alvarezii* were able to exhibit good radical scavenging activity. The results obtained were in accordance with those reported by (Neoh *et al*., 2016) of around IC_{50} - 9.55 -11.81 mg $ml^{-1,}$ and (Araujo *et al*., 2020) showed 7.15% to 31.21% at 5 mg ml^{-1} for a methanolic extract of *Kappaphycus alvarezii*.



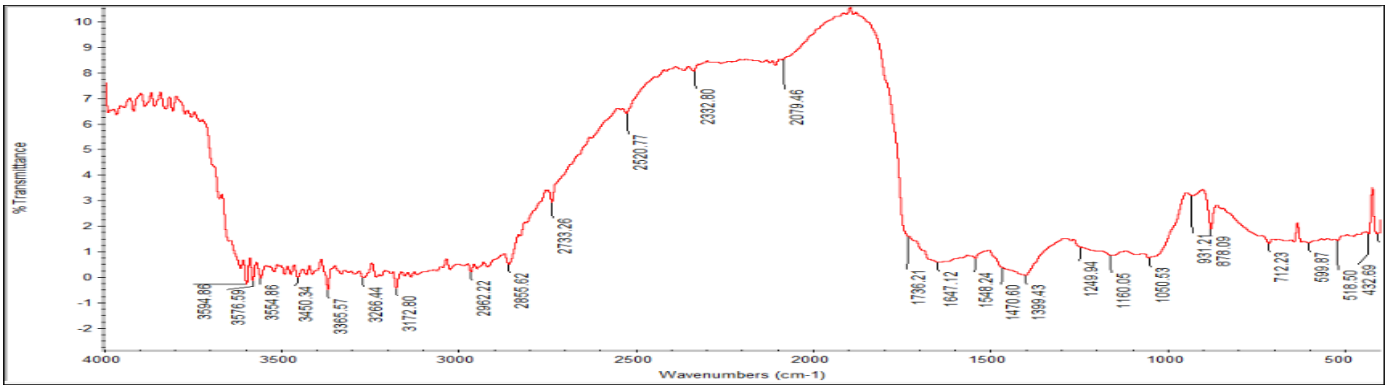
a. aqueous extract



b. 40% ethanolic extract



c. 60% ethanolic extract



d. 80% ethanolic extract

Fig 1. FTIR Graph of Kappaphycus seaweed extracts {a. aqueous extract, b. 40% ethanolic extract, c. 60% ethanolic extract, d. 80% ethanolic extract}

Table 1. Antioxidant properties of *Kappaphycus alvarezii* solvent extracts

Solvent extraction	Extraction yield (%)	TPC (mg gallic acid equivalent/g of extract)	TFC (mg quercetin equivalent /g of extract)	DPPH (IC_{50} mg/ml)
Aqueous	9.26 ± 0.039 ^{d}	9.87 ± 0.073^{a}	2.64 ± 0.104 ^{a}	9.6238 ± 0.03^{d}
40% ethanol	8.67 ± 0.002 ^{c}	10.98 ± 0.041^{b}	3.24 ± 0.152 ^{b}	7.9965± 0.043^{c}
60% ethanol	8.13 ± 0.001^{b}	12.82 ± 0.067^{c}	4.44 ± 0.07^{d}	6.5056 ± 0.076^{a}
80% ethanol	6.56 ± 0.019 ^{a}	12.70 ± 0.094^{c}	4.192 ± 0.100 ^{c}	7.0312 ± 0.033^{b}

Note: All the data were provided in Mean ± Standard deviation of three replicates (n=3). The values in the same column having different superscripts (a,b,c,d) are significantly different as determined by Tukey's post hoc test ($P < 0.05$)

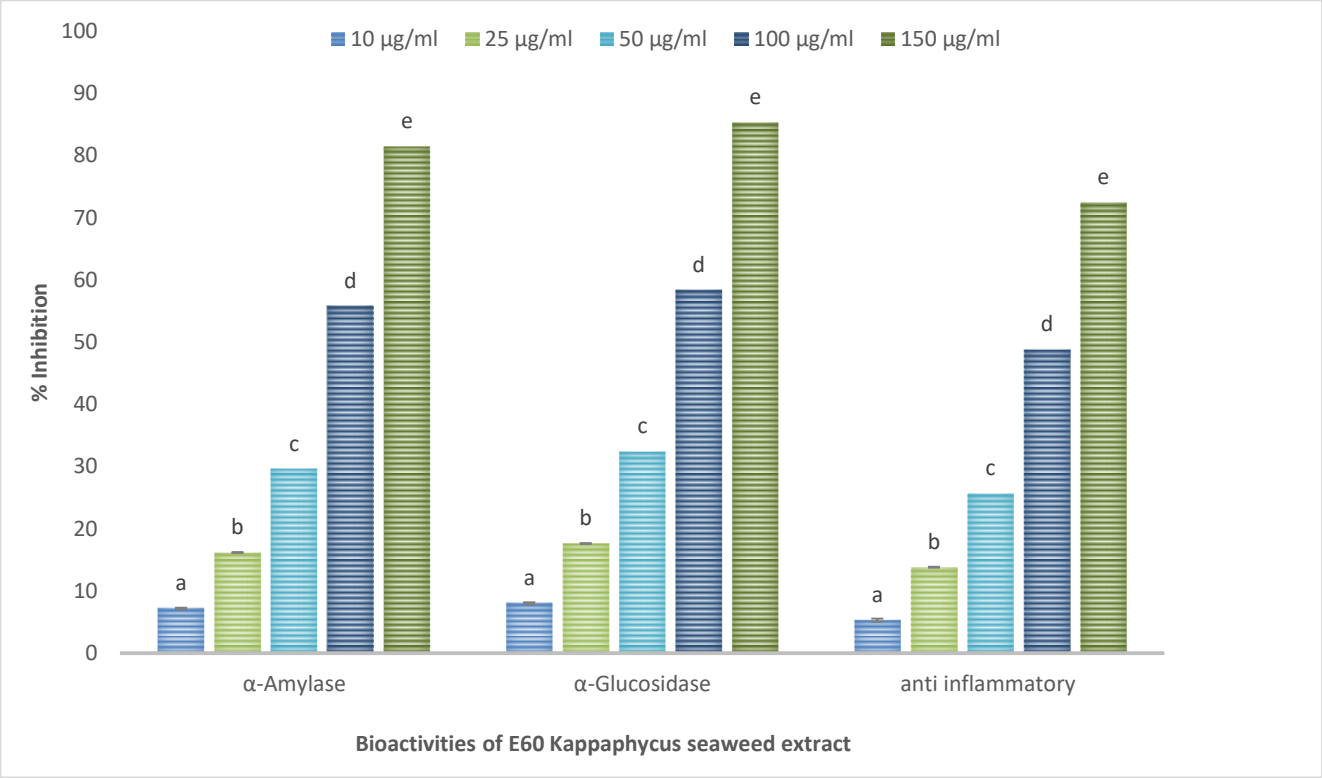
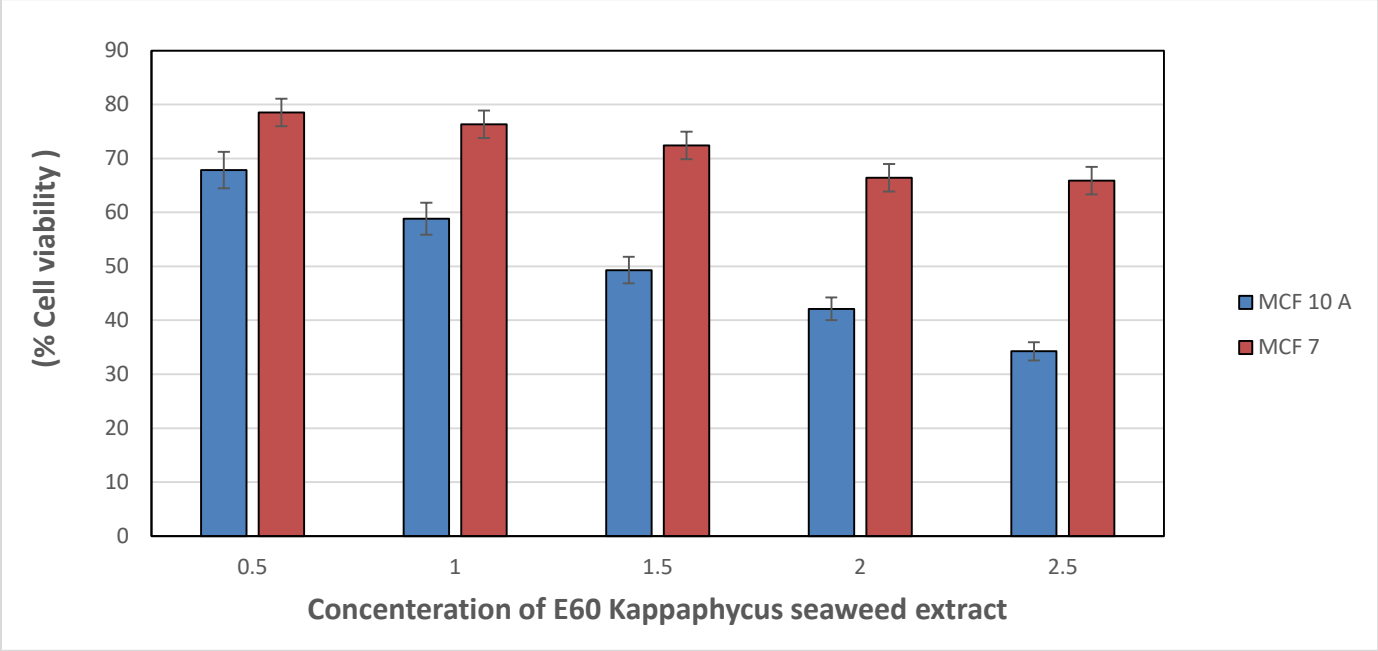


Fig 2. Antidiabetic and anti-inflammatory inhibitory activities from *K. alvarezii* (E60)

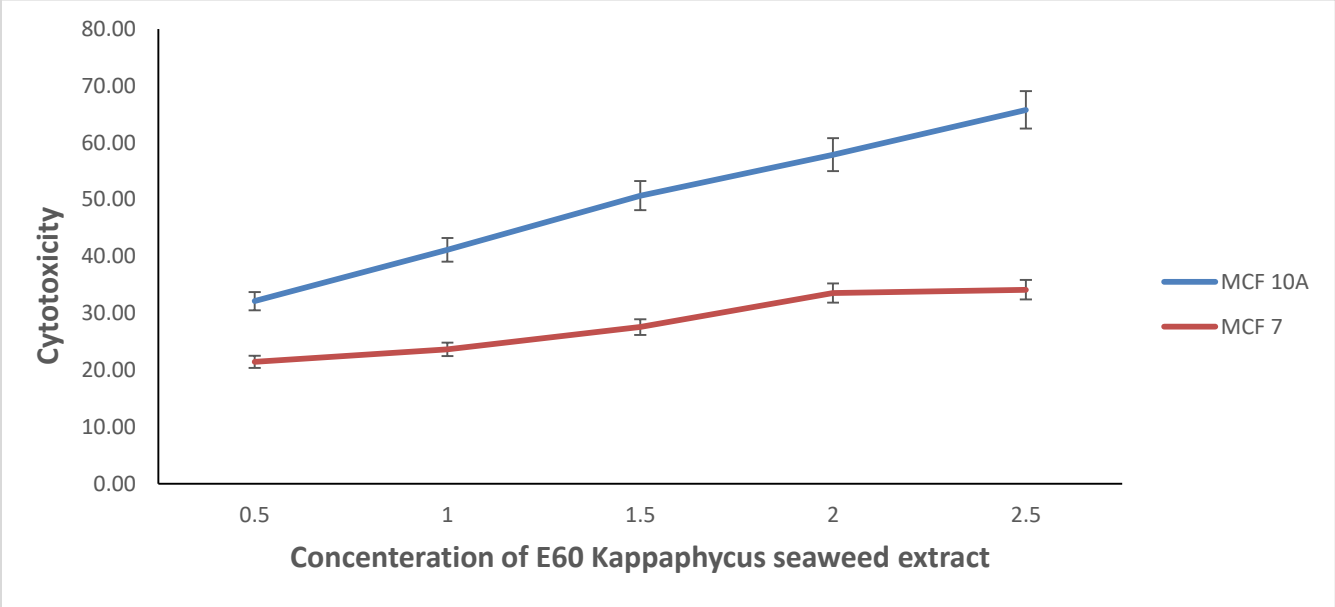
Table 2. IC_{50} Values of antidiabetic and anti-inflammatory activities of E60 extract

Bioactivities	E60 (*Kappaphycus alvarezii* (IC50 (mg/ml)
Antidiabetic activities	
α-Amylase	0.089 ± 0.01
α-Glucosidase	0.085 ± 0.01
Anti-inflammatory activity	
Inhibition of egg albumin denaturation	0.10 ± 0.01

Note: All the data were provided in Mean ± Standard deviation of three replicates (n=3).



3a. Cell viability (MCF 10 A and MCF 7 cells) against E60 extract



3b. Cell Cytotoxicity (MCF 10A and MCF 7) against E60 extracts
Fig 3(a, b). In vitro cytotoxicity of Kappaphycus seaweed extract

Diyana *et al*. (2015) reported that 50% ethanolic extract of *Kappaphycus alvarezii* showed higher antioxidant capacity with 19.35% ± 0.78% to that of 70% and 100% ethanol and (Kumar *et al*., 2008) reported a maximum scavenging activity in ethanolic extracts IC_{50} 3.03 mg ml^{-1} than water extract IC_{50} 4.76 mg ml^{-1}. The present study revealed that the extraction using ethanolic solvent exhibited maximum antioxidant activity with a similar correlation to the phenolic compounds.

Bioactive Properties
In vitro -Antidiabetic Activity

Diabetes mellitus is a serious health problem worldwide that occurs due to the inability of the human body either to insulin secretion, insulin action, or both.

The deficiency of insulin leads to hyperglycemia retinopathy, neuropathy, and nephropathy with interferences in carbohydrate, fat, and protein metabolism (Bearse *et al*., 2004; Makkar and Chakraborty, 2017). Nowadays, there is a paradigm shift towards the utilization of seaweed sources compared to synthetic hypoglycemic drugs. The bioactive compounds of seaweed particularly the polyphenol derivatives have the greatest ability to inhibit alpha-amylase and α- glucosidase enzymes through delay in carbohydrate hydrolysis, disaccharides, and glucose absorption and inhibition of sucrose metabolism into glucose and fructose due to the cleavage of α-D-(1-4) glycosidic bonds (You *et al*., 2012; Hamadan and Afifi, 2004; Confronty *et al.*, 2005). Different concentrations of ethanolic (E60) extract (0.01–0.15 mg/ ml) were tested for α-amylase and α-glucosidase inhibitory activity of *Kappaphycus alvarezii* shown in table 2 and fig 2.

The reduction of amylase and glucosidase enzyme activity is solely depending upon the concentration. The seaweed extract exhibited similar α-amylase inhibitory activity (IC_{50} –0.089 mg/ml) and α- glucosidase inhibitory activity (IC_{50} -0.084 mg/ ml) as the results were in accordance with (Makkar and Chakraborty, 2016) having lower to the inhibitory activity of α-amylase obtained from sulfated galactans of *Kappaphycus alvarezii* (IC_{50} 0.15 mg/ml) and similar α-glucosidase inhibitory activity (IC50-0.09 mg/ml). The ethanolic extract of *Eucheuma cottonii* showed the highest inhibition activity of α-amylase (59.33%) for greater than 0.2 mg/m compared to the aqueous extract (Prasasty *et al*., 2019). The crude extract obtained from *Caulerpa racemosa* and *Spatoglossum schroederi* using acetone exhibited α-amylase activity, IC_{50} of 0.09 mg/ml and 0.58 mg ml, respectively (Teixera *et al*., 2007). Thus, the present study proved that seaweed extracts are a rich source of antidiabetic agents as the presence of bioactive properties is mainly due to the presence of sulfated polysaccharides, phenolics and terpenoids

In vitro -Anti-inflammatory Activity

Inflammation is a defense phenomenon occurred mainly due to injury, harm, and contagion or it may also be obtained due to the release of chemical substances from migrating cells (Vaughan *et al*., 2013).

Red seaweeds contain many bioactive metabolites in contrast to other seaweed classes (Frias *et al*., 2010). The treatment of human skin diseases can be done by using anti-inflammatory agents. Among widespread marine resources, seaweed is shown to possess anti-inflammatory activities (Vairappan *et al*., 2001; Xu *et al*., 2003; Mayer and Hamann, 2005). The process of albumin denaturation helps in measuring the protein denaturation as, during the denaturation process, most biological proteins will lose their function. The denaturation of proteins is one of the causes of inflammation (Leelaprakash and Mohan Das, 2011).

The Mechanism of the assay in which the egg albumin induces heat to denature proteins, in the present study, the ability of E60 seaweed extract to inhibit protein denaturation was studied and compared with a standard drug aspirin, a well-known anti-inflammatory drug that was used as a positive control illustrated in table 2 and fig 2. The maximum inhibitory effect was obtained at 72.4% for 0.15 mg/ml. The seaweed extract exhibited inhibitory activity at IC_{50} value 0.105 ± 0.01mg/ml. Ali *et al*. (2019) reported anti-inflammatory activity from *Thalassiosira weissflogii* inhibiting albumin denaturation with a maximum inhibition of 83.72% at 500 µg/ml as it acts as a proteinase inhibitor, and prevents denaturation. Makkar and Chakraborty (2017) reported the anti-inflammatory activity of sulfated polysaccharides against 5- lipoxygenase (5-LOX) and cyclooxygenase inhibition assay with IC_{50} of 0.34 mg/ml and 0.06 mg/ ml respectively. Hence, the presence of the rich source of antioxidants such as phenolic compounds and their derivatives in *K. alvarezii*, has the ability in the wound healing process and acts as an anti-inflammatory agent to prevent tissue damage (Jennifer *et al*., 2015; Selvan *et al*., 2014).

In vitro Toxicity Assays

In vitro toxicity testing methods are mostly preferred over In vivo toxicity studies due to more time and cost-effective methods**.** MTT cytotoxicity assay is the fastest indirect calorimetric assay for determining the viability of breast adenocarcinoma (MCF7) and Breast epithelial cells (MCF 10-A) when exposed to different concentrations of E60 kappaphycus extract. In this study, the viability of cells was reduced based on the concentration after 24 hours of incubation as shown in fig 3 a & b.

The study is based on the ability of live but not dead cells to convert yellow tetrazolium bromide to the purple formazan derivative by mitochondrial succinate dehydrogenase enzyme in living cells and the amount of formazan is directly proportional to the number of viable cells due to the metabolism of -(4, 5- dimethylthiazol-2-y1)-2, diphenyltetrazolium bromide (MTT) salt. The dose-response of the different concentrations of seaweed extracts (0.5 - 2.5 mg/ml) was observed against two cells (MCF-10A and MCF-7 cells) and the results were shown in fig 4. The results exhibited a higher decrease in the viability of the cells treated at the highest concentration (2.5 mg/ml) of Kappaphycus 60% ethanolic extracts (65.75 % death in MCF-10 A cells and 34.13 % in MCF-7 cells). The strong cytotoxicity was found with IC_{50} value of 1.528 mg/ml. The mechanism of cytotoxicity occurs due to several mechanisms as the anticancer cells shows cytoxicity in cells either by activation of apototic route or through a cytostatic effect that stops the cellular cycle (Lundberg and Weinberg, 1999). Comparatively the polar solvents like ethanol, methanol, ethyl acetate and petroleum ether extracts of seaweeds possess cytotoxic properties**.** The results were reported similar by (Papitha *et al*., 2020 on the cytotoxicity capacity of Kappahycus extract using methanol solvents with IC_{50} value of 1.75 mg/ml against HeLa cell lines. Marine algae indicated that they are potentially useful in therapeutic and pharmaceutical purposes by simultaneous activation of different apoptotic factors and pathways (Kim *et al*., 2010).

Conclusion

In the present study, the comparative assessment of the extraction efficiency of solvents using different gradients of ethanol and water was carried out and the results revealed that the extract possessed variations in extraction yield, total phenolics, flavonoid and antioxidant activity. Among the various extracts, 60% ethanolic was obtained as an effective solvent for obtaining antioxidant rich extract with high phenolics and assessed for bioactive properties such as In vitro antidiabetic activity, anti-inflammatory activity and cytotoxic activity. The significant findings from the study suggested that seaweed extracts can be utilized as an effective source of natural antioxidants in food and pharmaceutical sectors.

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